ABSTRACT

CHEN, HUI. Process Development and Fundamental Study on Enzymatic Hydrolysis of Cellulosic Biomass to Fermentable Sugars for Ethanol Production. (Under the direction of Dr. Richard Venditti and Dr. Hasan Jameel).

Waste paper materials such as office paper, newspaper and paper sludge present unique advantages for bioethanol production. They are abundant, low lost and require less intensive pretreatment to open up the lignocellulosic structure for enzymatic hydrolysis. The objective of this research is to develop conversion processes that are technically and economically feasible for producing ethanol from waste paper materials. Identifying and understanding the inhibition factors in lignocellulosic biomass and enzymes that influence enzymatic hydrolysis of fermentable sugars are also covered. A fundamental study of the effects of pretreatment and lignin removal on softwood was also conducted because softwood is a major wood resource in many parts of the world and a major source of paper materials.

Different types of waste paper materials were studied in this research in terms of their feasibility for fermentable sugar production via enzymatic hydrolysis. In **Chapter 2**, low enzyme dose on recovered office paper was investigated. Ash removal was identified to be necessary since both acid soluble and acid insoluble ash adsorbed enzyme during enzymatic hydrolysis. This ash-enzyme interaction was proven to have higher affinity than cellulose-enzyme interaction. The effect of hornification - irreversible pore collapse in lignocellulosic fibers was also studied. Mechanical refining by a PFI mill of previously dried fibers improved sugar recovery to similar or higher levels as never dried fibers.

Paper sludge is another attractive biomass source for the conversion to ethanol. A mechanical fractionation process was proposed in **Chapter 3** in order to remove ash from

sludge prior to enzymatic hydrolysis. This process removed 82-98% of the ash with fiber yields from 39-69%. Fractionation efficiency was also evaluated by testing different size mesh screen openings, aiming to optimize this fractionation process. The ash rich streams had a lower C:N ratio than the original sludge, which improved its suitability as soil amendment.

In **Chapter 4**, process simulation using engineering process simulation software WinGEMS and financial analysis on the feasibility of the process developed in Chapter 3 were conducted. The financial impact of the addition of the sludge fractionation step was discussed based on using sludge from virgin and recycled paper mills. The most profitable case was fractionated virgin sludge (from a virgin paper mill) to ethanol (F-VK1) with a net present value (NPV) of US$ 11.4 million, internal rate of return (IRR) of 28%, payback period of 4.4 years and minimum ethanol revenue (MER) of US$ 0.32 per liter. Risk analysis showed that the F-VK1 case obtained a near 100% probability of business success with both optimistic and pessimistic assumptions.

Newspaper contains high lignin content among various waste paper materials. **Chapter 5** is focused on developing a pretreatment process ideal for newspaper saccharification. The effects of non-ionic surfactant and flexo link were also studied. Tween 80 improved sugar conversion of newspaper and flexo ink was proven to have no inhibition effects on enzymatic hydrolysis. Pretreatment including autohydrolysis, mechanical refining, oxygen, alkaline and green liquor (GL) pretreatments were evaluated on newspaper. Except mechanical refining and oxygen pretreatment, all the other pretreatment methods adversely affected enzymatic hydrolysis of newspaper. It was presumably due to the pore collapse in the fibers during the pretreatment process.

Softwoods are the dominant lignocellulosic feedstocks in the Northern hemisphere and viewed to be attractive and sustainable for ethanol fuel production. However, lignocellulosic biomass, especially softwood, is recalcitrant to biological degradation due to its rigid and compact structure, as well as its chemical compositions. In **Chapter 6**, a fundamental study on the effect of lignin removal by different pretreatments was performed. Lignin by oxidative treatment yielded higher enzymatic hydrolysis efficiency than kraft pulping and this was especially significant with sodium chlorite pretreatment. Factors affecting softwood saccharification were investigated by generating softwood fibers with the same bulk lignin content (11.3%-13.7%) by kraft pulping, sodium chlorite, oxygen and high consistency ozone delignification. Lignin hydrophobicity was identified as one of the most important factors influencing enzymatic hydrolysis of softwood.

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Process Development and Fundamental Study on Enzymatic Hydrolysis of Cellulosic Biomass to Fermentable Sugars for Ethanol Production

by
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DEDICATION

I would like to dedicate this dissertation to my parents, Xiaoyu Zhang and Weiyuan Chen, who have supported and encouraged me to seek the pleasures of life, and to my dearest late grandmother Yafen Liu, whose love and wisdom I will forever treasure.

BIOGRAPHY

Hui Chen was born on March 12, 1987 in Yangzhou, Jiangsu province, China. She received her Bachelor of Engineering in Light Chemical Engineering from Nanjing Forestry University in June of 2009. In her junior year, Hui went to Finland as an exchange student and studied in the Paper Technology Degree Program, Tampere University of Applied Sciences, Tampere, Finland for one year and during her stay, she acquired a summer internship at the headquarters of UPM Raflatac. Her positive experiences and passion to seek new knowledge within the pulp and paper field influenced her to pursue graduate studies in the United States.

She began her Ph.D. studies in August of 2009 in the Department of Forest Biomaterials at North Carolina State University under the guidance of Dr. Richard Venditti and Dr. Hasan Jameel. During her Ph.D. career, Hui worked for Novozymes North America and Domtar Plymouth pulp mill. She was later offered a permanent position at Andritz Automation.

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Chapter 1 Literature Review

Overview of cellulosic biomass to ethanol

Cellulosic biomass, the most abundant renewable material on Earth, has been identified to have great potential as a source of raw materials for energy production in the forms of heat, steam, electricity and liquid biofuel such as ethanol (Sims et al., 2006; Sun & Cheng, 2002). Ethanol can be used directly as fuel or blended with gasoline (e.g., E10 [gasoline containing 10% ethanol]). It is an oxygenated fuel containing 35% oxygen, which has a clean burning characteristic that reduces particulate and NO_x emissions from combustion (Lynd et al., 1991). Due to the rise of the nation's gasoline price in part caused by the dependence on imported petroleum, a considerable amount of research interest pertaining to alternative energy sources have been triggered (Shi et al., 2009). One of the most promising alternatives for the replacement or enhancement of conventional fossil fuels is to utilize liquid ethanol fuels produced from cellulosic biomass or biomass-derived products (Ballesteros et al., 2002).

Lignocellulosic biomass to ethanol includes substrates from woody and non-woody biomass, paper wastes and agricultural residues. The two main categories of woody biomass include softwood and hardwood. Non-woody biomass includes crop residues (straw, leaves, and plant stems) and grasses such as wheat straw, corn stover, corn cobs, sugarcane bagasse, sweet sorghum, rice straw, Coastal Bermuda grass, switch grass, bamboo etc. (Sathitsuksanoh et al., 2010; Sun & Cheng, 2002). Most substrates categorized as non-woody biomass may also fall into the agricultural residue category. The final category includes paper wastes, which can be obtained from waste paper materials in municipal solid waste

(MSW), commercial and industrial wastes such as sawdust, wood chips, paper, packing materials, textiles, paper industrial sludge etc. (Duff & Murray, 1996).

The main steps in ethanol production from biomass are as follows: hydrolysis of cellulose and hemicellulose in the lignocellulosic materials to fermentable reducing sugars, sugar fermentation to ethanol, separation of process residue (mainly lignin) before or after fermentation and, finally, recovery and distillation of ethanol to meet fuel specifications (Alvira et al., 2010). Cellulase enzymes are applied during the hydrolysis step, providing a mild process environment compared to processes like acid hydrolysis. The nature of biomass itself hinders cellulase enzymes from digesting cellulose due to physical and chemical barriers including lignin, hemicellulose and other compositions. Thus, for most types of biomass, pretreatment is necessary prior to enzymatic hydrolysis in order to break down the lignocellulosic structure by removing lignin and hemicellulose, reducing cellulose crystallinity, and increasing the porosity of the materials (Sun & Cheng, 2002).

Pretreatment of biomass to ethanol

An ideal pretreatment for lignocellulosic biomass should provide the following desirable properties (Lynd, 1996; Sun & Cheng, 2002): (a) produce accessible and reactive fibers, (b) yield carbohydrates in non-degraded form, (c) avoid formation of carbohydrate- and lignin-derived byproducts that inhibit subsequent hydrolysis and fermentation, (d) produce no solid residues, (e) work in reactors of reasonable size with moderate cost, (f) be cost-effective with a high degree of simplicity, and (g) be effective at low moisture contents. The categories of available pretreatment options are: physical, physico-chemical, chemical, and biological

processes. Since different lignocellulosic materials have different physico-chemical characteristics, it is necessary to adopt suitable pretreatments technologies based on the lignocellulosic biomass properties of each raw material.

Physical pretreatment

The purpose of physical pretreatment is to mechanically reduce biomass particle size, cellulose crystallinity and degree of polymerization as well as increasing specific surface area. Physical approaches on biomass to ethanol that have been studied involve mechanical size reduction, internal delamination and surface fibrillation such as chipping, grinding, milling, extrusion, refining, pyrolysis etc. (Jones et al., 2013; Karunanithy & Muthukumarappan, 2010; Sun & Cheng, 2002). In addition, irradiation by gamma ray, electron beam and microwave have also been evaluated (Taherzadeh & Karimi, 2008). Generally, chemicals are not required when doing physical pretreatment. However, most methods are high energy-demanding with little lignin removal. Hence, physical pretreatment like refining is combined with chemical pretreatments (usually as a post-treatment) to lower the overall energy and chemical demand, while still achieving desirable conversions without too much sugar degradation (Chen et al., 2012; Wu et al., 2010).

Physico-chemical pretreatment

Physico-chemical pretreatment usually refers to the explosion method for pretreatment of lignocellulosic materials. Steam explosion is one of the common and simple methods that involves only water with a reaction temperature ranging from 160 to 260 ℃ (Sun & Cheng, 2002). This process mainly converts hemicellulose into soluble oligomers with little impact

on lignin degradation (Fernandez-Bolanos et al., 2001). It also generates byproducts such as acetic acid (hydrolysis of acetyl groups linked to sugars), furfural (product of pentose dehydration – xylose and arabinose) and hydroxymethyl furfural (HMF) (dehydration of hexoses – glucose, galactose and mannose in this case) (Gámez et al., 2006). Furfural could be further degraded into formic acid and HMF into formic and levulinic acid (Taherzadeh & Karimi, 2007). Since these degradation products are inhibitory to enzymatic hydrolysis, and fermentation, a washing step is necessary to remove the inhibitory materials prior to enzymatic hydrolysis (Sun & Cheng, 2002). Autohydrolysis or liquid hot water (LHW) is another pretreatment that does not require chemicals. Compared to stream explosion, autohydrolysis is conducted at a similar temperature range of 130 to 260 °C (Lee et al., 2010b). However, it requires higher water demand and does not require rapid decompression. As a result, higher hemicellulose recovery and lower inhibiting byproducts are obtained (Alvira et al., 2010).

To improve pretreatment efficiency, steam explosion with the addition of chemicals such as H_2SO_4 (or SO_2), CO_2 and ammonia fiber explosion (AFEX) have been evaluated (Sun & Cheng, 2002). SO_2 explosion is a method using either SO_2 or H_2SO_4 impregnation prior to steam explosion at a temperature of 160 to 230 °C with the aim of mainly degrading hemicellulose (Eklund et al., 1995). It was reported that the addition of SO_2 yielded higher glucose compared to H_2SO_4 even though higher hemicellulose loss occurred (Eklund et al., 1995). CO_2 explosion is based on the utilization of CO_2 as a supercritical fluid to effectively remove lignin and disrupt cellulose structure with low inhibitor formation (Alvira et al.,

2010). AFEX is a kind of alkali physico-chemical pretreatment process where the biomass is exposed to liquid ammonia at ~90-100 ℃ for a certain period of time (e.g. 30 min) and followed by an immediate reduction of pressure, leaving only the pretreated solid material (Taherzadeh & Karimi, 2008). The AFEX process does not significantly solubilize hemicellulose and can partially remove or modify lignin fraction. Although inhibitory byproducts are not produced in this process, washing is still recommended due to the existence of lignin fragments and cell wall extractives on the cellulose surface. In addition, the AFEX method was reported to have little effect on substrate with high lignin content (above 20%) and the cost of the process is also relatively high (Sun & Cheng, 2002).

Chemical pretreatment

Acid pretreatment

Inorganic acids such as H_2SO_4, HCl and HNO_3 and organic acids such as fumaric and maleic acid have been used to treat lignocellulosic materials. Early studies show that concentrated acid (30-70%) at low temperature (e.g. 40 ℃) is effective in terms of breaking down lignocellulosic structure and achieving high sugar conversion (Sivers & Zacchi, 1995). However, interest has been reduced in this process due to corrosive conditions, high cost on non-metallic or alloy equipment and the challenge of acid recovery. Despite these drawbacks, a few projects are still proceeding using this technique (Taherzadeh & Karimi, 2007).

Dilute acid pretreatment using H_2SO_4 appears to be more favorable for practical application and have been studied for pretreating a wide range of lignocellulosic biomass materials. This is typically conducted using low acid concentration (e.g. 0.1-1% H_2SO_4) and

can be performed at lower temperatures (less than 160 ℃) for longer periods of time (30-60 min); or at higher temperature (e.g. 160-240 ℃) for shorter periods of time (e.g. 10 min) (Taherzadeh & Karimi, 2007). The mechanism behind this process is the solubilization of hemicellulose, mainly xylan, and also converts solubilized hemicellulose to fermentable sugars. It is not effective in lignin removal, but can disrupt lignin to a certain extent to increase enzyme accessibility to cellulose (Yang & Wyman, 2004). High hydrolysis yields have been reported when pretreating lignocellulosic biomass with diluted H_2SO_4. However, several draw-backs are present in the dilute acid pretreatment method. First, the pH of the process stream following pretreatment must be neutralized to the optimum range of enzymes for hydrolysis and further adjustments are necessary for fermentation. This process also suffers from low yields because of sugar decomposition. Sugar degradation in a single stage dilute acid pretreatment process tend to form furfural, HMF, carboxylic acids, furans and phenolic compounds, which are potentially inhibitory to fermentation (Taherzadeh & Karimi, 2008). In order to reduce sugar degradation, studies have been done using two or more stages of dilute acid hydrolysis with mild conditions in the first stage and more severe conditions in the subsequent stage(s) (Taherzadeh & Karimi, 2007).

Alkali pretreatment

Alkali pretreatment refers to the utilization of base chemicals in the pretreatment process. A wide range of alkaline chemicals have been studied for pretreatment use such as: NaOH, $Ca(OH)_2$ (lime), Na_2CO_3, $NaHCO_3$, green liquor (Na_2CO_3+Na_2S), ammonia and furthermore, SO_2 in alkaline conditions have also been applied (Jin et al., 2010; Taherzadeh & Karimi,

2008). The mechanism behind the alkali pretreatment process is to swell lignocellulosic materials, causing disruption in lignin structures and linkages between lignin and carbohydrates. An increase of internal surface area and decrease in cellulose crystallinity and degree of polymerization was observed (Sun & Cheng, 2002). Alkali pretreatment has been shown to cause less sugar degradation than acid pretreatment and it was reported to be more effective on non-woody (agricultural residues) than on woody biomass (Alvira et al., 2010). There is also a consensus that dilute alkaline pretreatment is more effective on hardwood than softwood (Sun & Cheng, 2002; Wu et al., 2010).

To achieve higher lignin removal, alkali pretreatment can be combined with ammonia, O_2 or H_2O_2 at temperature of ~100-220 ℃. High sugar yields in enzymatic hydrolysis with effective lignin removal (40-85%) has been reported in these alkali based wet oxidation techniques (Iyer et al., 1996; Koo et al., 2011; Martin et al., 2007; Saha & Cotta, 2007).

Ozonolysis

Ozonolysis pretreatment by ozone is very effective in degrading lignin in a short period of time. It can be performed in high (e.g. 35%) or low (e.g. 1%) substrate consistency. Since ozone reacts with lignin in the gaseous phase, it was reported that ozone pretreatment conducted with high substrate consistency was more effective than with low consistency (Neely, 1984). Neither high temperatures nor pressures are involved in the ozonolysis pretreatment process and inhibitory residues are not generated. Thus the process is exceedingly well suited for scaling-up or scaling-down with design simplicity. However, the

main drawback of ozonolysis is the large amount of ozone required that makes this process economically undesirable (Alvira et al., 2010).

Ionic liquids (ILs) pretreatment

ILs (e.g. 1-ethyl-3-methylimidazolium acetate) are salts, typically composed of large organic cations and small inorganic anions, which exist as liquids at wide ranges of temperatures (usually lower than 100) (Alvira et al., 2010). Due to their high thermal stability and nearly complete non-volatility, non-flammability, low vapor pressures and non-toxicity, ILs can be used as a "green solvent" to break the internal lignin and hemicellulose bonds (Lee et al., 2009). In addition, ILs pretreatment is one of the few processes that enable lignin and hemicellulose solubilization and recovery. A lower degree of crystallinity on dissolved cellulose was observed using this process. However, some concerns that ILs residue on the substrate may negatively affect enzyme and yeast performance. Development of energy efficient recycling methods for ILs is a prerequisite for both economic and technical concern since ILs recovery is necessary for cost reduction. Due to the immaturity of this technique and high cost of overall operation, this process is still commercially unavailable (Alvira et al., 2010).

Organosolv

Organosolvation is a pretreatment process utilizing organic or aqueous solvent mixtures to solubilize lignin and possibly a part of hemicellulose that results in easier access to cellulose for enzymatic hydrolysis (Pan et al., 2006). A number of solvents can be used including methanol, ethanol, acetone, ethylene glycol and tetrahydrofurfuryl alcohol. Acid

catalysts (e.g. HCl, H_2SO_4, oxalic or salicylic) have been studied in order to lower the reaction temperature and obtain high yields of xylose. High temperatures (above 185 ℃) facilitates delignification to an ideal extent that acid catalysts become unnecessary (Alvira et al., 2010). One advantage of organosolv pretreatment is that this process produces two relatively pure byproducts, high-quality reactive lignin and an aqueous hemicellulose stream. However, the organosolv pretreatment process is relatively more expensive than steam explosion and the commercial feasibility highly depends upon complete recovery of the organic solvents (Duff & Murray, 1996).

Biological pretreatment

Among all the methods, fungal pretreatment provides the mildest environmental condition with low capital and energy cost, no chemical requirements and unit operation simplicity. Microorganisms such as brown, white and soft-rot fungi have been used. Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin (Sun & Cheng, 2002). Lignin degradation by white-rot fungi was found to be the most effective for biological pretreatment of lignocellulosic materials. It degrades lignin and hemicellulose with major cellulose preservation (Shi et al., 2008). The major drawbacks for moving this biological pretreatment forward is the low hydrolysis rate in the processes (Sun & Cheng, 2002). Thus, biological study of more basidiomycetes fungi or even genetic modification can enable more efficient fungi delignification.

Inhibitions of biomass to ethanol

Main factors that influence enzymatic hydrolysis of lignocellulosic biomass can be divided into two groups: enzyme-related and substrate-related factors. Many of the factors are also interrelated during the hydrolysis process. Thus, in studies on factors affecting enzymatic hydrolysis of biomass, it is often impossible to change one factor without altering others. This is especially true for substrate-related factors, though these factors are discussed separately.

Enzyme-related factors

The kinetics of cellulase digestion consist of three steps: adsorption of cellulase enzymes onto the surface of the cellulose, digestion of cellulose to fermentable sugars, and desorption of cellulase (Sun & Cheng, 2002). For the same enzyme, dosage and activity are the two most influential factors that affect enzymatic hydrolysis efficiency. Increasing the dosage of enzymes, to a certain extent, could improve the hydrolysis rate and sugar conversion. However, the financial feasibility of any biomass to ethanol project using an enzymatic approach highly depends upon the cost of the enzymes. Under current status of the technology and the market, a cellulose dosage of ~5FPU/g cellulose is a reasonable loading to possibly obtain positive financial returns, though many other factors in a financial model also play important roles (Chen et al., 2014).

Inhibitions of enzyme consist of three sources: internal, external and product. Internal inhibitions in a complex cellulase system include cellobiose inhibition of endo- and exo-glucanases and glucose inhibition of β-glucosidases. External inhibitions can come from

enzymatic hydrolysis conditions due to shaking, shearing and gas/liquid interface interactions. Besides enzyme–substrate interaction, competitive and noncompetitive inhibitions caused by enzyme–product interaction also contribute to the deactivation of enzymes (Gan et al., 2003).

It has been reported that improving enzyme adsorption-desorption activity and avoiding irreversibly inert binding are the keys to improving enzymatic digestibility (Sun & Cheng, 2002). Non-ionic surfactants such as Tween 20, 80 are therefore used to improve the rate of enzyme hydrolysis and sugar conversion at the same enzyme dosage (Eriksson et al., 2002).

Substrate-related factors

There is consensus, but also a rich diversion of views of substrate-related factors influencing enzymatic hydrolysis and many of them are interrelated. For example, substrate particle size has a strong influence on initial reaction rate (Gan et al., 2003) and final sugar yield was also reported to increase with particle size reduction (Yeh et al., 2010). In addition, it was claimed that there lies an optimum range for particle size and further particle size reduction causes a decrease in enzymatic conversion (Duff et al., 1995). With regard to substrate concentration, lower concentrations in enzymatic hydrolysis allow for higher sugar conversions. However, more interests have been raised on high solid enzymatic hydrolysis, with the aim to increase sugar concentration, save energy and capital investments for downstream operations (Kristensen et al., 2009).

Crystallinity

Cellulose microfibrils contain crystalline and amorphous regions. Crystallinity is given by the amount of crystalline fraction in the substrate matrix. The crystallinity index (CrI) can

be calculated using wide-angle X-ray diffraction. CrI measurement is highly affected by factors such as the drying conditions prior to measurement of CrI and residual cells and proteins (Zhang & Lynd, 2004). There is discrepancy in terms of the effects of crystalline on enzymatic hydrolysis. It is well acknowledged that decreasing the crystallinity increases the initial hydrolysis rate and even final sugar yields (Mittal et al., 2011; Sinitsyn et al., 1991; Yoshida et al., 2008). However, other evidences indicate crystalline does not necessary correlate to the final yield of enzymatic hydrolysis. Some studies show that an increase in crystallinity of pretreated material did not negatively affect the yield of enzymatic hydrolysis. In one such case, crystallinity was increased by certain pretreatments such as ball milling and sugar conversion was improved (Fan et al., 1980; Kim & Holtzapple, 2006). Due to the uncertainty of methodologies for measuring CrI and conflicting results on the change of CrI during hydrolysis as well as the discrepancy regarding crystallinity-sugar conversion correlation, it is difficult to conclude at this time that CrI is a key determinant in the rate of enzymatic hydrolysis (Yu et al., 2011; Zhang & Lynd, 2004).

Degree of polymerization

The degree of polymerization (DP) determines the relative abundance of terminal and interior β-glucosidic bonds, and of substrates for exo-acting and endo-acting enzymes. It is essentially related to other substrate characteristics, such as crystallinity. The DP of cellulosic substrates varies greatly (from <100 to >15000) (Zhang & Lynd, 2004). The DP of wood is typically from 4000 to 5000 and after pulping, the DP is reduced to 500-2500 (Miksche, 1980; Zhang & Lynd, 2004). In enzymatic hydrolysis, endoglucanases cut the interior

portions of the cellulose chains, preferentially less ordered, being primarily responsible for decreasing the degree of polymerization of cellulosic substrates. Exoglucanase has been found to have a marked preference for substrates with lower DP. Many studies, from the late 1970s till recent, show a positive correlation between sugar yield and DP (Ha et al., 2011; Okazaki & Moo-Young, 1978; Puri, 1984). However, experimental data indicates that the DP of some substrates remained nearly constant during the later phase of hydrolysis, yet the sugar conversion was improved (Cateto et al., 2011; Zhang & Lynd, 2005).

Accessible surface area (pore volume)

Enzymatic hydrolysis is the heterogeneous catalytic reaction that requires direct physical contact between enzyme molecules and cellulose. As a result, accessible surface area of cellulose is an essential factor in enzymatic hydrolysis. Accessible surface area is difficult to isolate as a single factor in natural lignocellulosic biomass since it is related to lignin, hemicellulose removal and particle size (Chandra et al., 2007). It has been widely studied that the rates and the extent of hydrolysis of pulp fibers are directly correlated to their initial specific surface area (Koo et al., 2011; Yu et al., 2011). One goal for any chemical or mechanical pretreatment is to increase the specific surface area for better enzyme digestion. The importance of surface area of biomass was proven by studies on fiber delamination and enhanced swelling that results from mechanical treatment (Chen et al., 2012). Recycled pulps originate from fiber sources that undergo irreversible changes in their structure upon drying. This phenomenon is often referred to as “hornification” - irreversible closure of pores in fibers upon drying- and has been proven to decrease sugar conversion in enzymatic

hydrolysis (Chen et al., 2012). The reduction in sugar yield was brought back by mechanically refining the hornified fiber. Such studies confirm that surface area is a rate limiting factor since no other compositional parameters in lignocellulosic materials have been altered. However, conclusion on pore volume affecting enzymatic hydrolysis may only be drawn on the same type of lignocellulosic material undergoing the same type of pretreatment. Due to the heterogamous nature of lignocellulose and various measurement methods, accessible surface area should be compared with caution.

Hemicellulose

It has been widely accepted that, similarly to lignin, hemicellulose acts as a barrier within the lignocellulosic matrix restricting access of cellulases to cellulose (Chandra et al., 2007). Many pretreatment methods mentioned above were shown to be able to remove hemicelluloses and consequently improve enzymatic hydrolysis. However, most pretreatments remove lignin as well and create higher pore volume/accessible surface area. Therefore, hemicellulose removal as a leading role for improving enzymatic hydrolysis cannot be established. In addition, current commercial enzyme cocktails enable hemicellulose hydrolysis by hemicellulase so that maintaining the hemicellulose in either a solid or liquid phase during pretreatment could maximize sugar recovery for the process.

Lignin

Lignin is by far the most abundant substance composed of aromatic moieties in nature (Calvo-Flores & Dobado, 2010). It is a complex phenylpropanoid polymer responsible for integrity, structural rigidity, and prevention of swelling of lignocelluloses. The main

substituents of lignin, p-coumaryl (p-hydroxyphenyl, H unit), coniferyl (guaiacyl, G unit) and sinapyl (syringyl, S unit) alcohol (Fig. 1.1), are polymerized by an enzyme-catalyzed dehydrogenative reaction (Freudenberg, 1965). Hardwood contains 18-25% lignin which is mainly composed of G and S units with trace amounts of H monolignols. Softwood contains 25-35% lignin and is predominantly made up of G units. Herbaceous plants/residues such as grass, straw and cobs contain ~5-15% lignin, with all three units in different ratios (Sun & Cheng, 2002; Zhao et al., 2012).

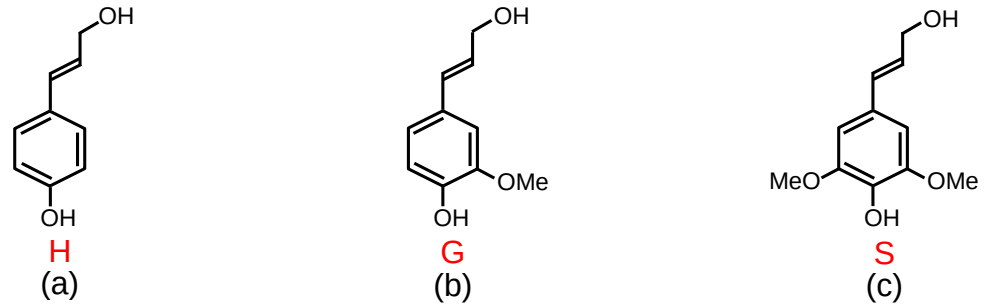

Fig. 1.1 Three types of monolignol: (a) p-coumaryl alcohol (4-hydroxyphenyl (H) unit), (b) coniferyl alcohol (guaiacyl (G) unit), (c) sinapyl alcohol (syringyl (S) unit)

Lignin removal and/or redistribution have been corroborated to have a significant effect on sugar conversion during enzymatic hydrolysis in many researches (Nakagame et al., 2010; Öhgren et al., 2007; Yoshida et al., 2008). The inhibition of lignin on enzymatic hydrolysis can be summarized into three aspects: physical barrier, inert adsorption of enzymes and enzyme deactivation by soluble lignin.

The physical/steric hindrance of cellulose was reported and can clearly inhibit sugar yield in enzymatic hydrolysis since the prerequisite for enzyme digesting cellulose is through

direct physical contact (Nakagame et al., 2010; Zhao et al., 2012). Besides the bulk lignin content, other important lignin characteristics have been recognized and studied (Chandra et al., 2007; Santos et al., 2012). These factors include the diversity of structures in lignin composition, hydrophobicity of lignin, as well as the distribution of lignin throughout a fiber. It is widely acknowledged that softwood is more recalcitrant to enzymatic hydrolysis than hardwood, even with the same lignin content (Mansfield et al., 1999; Yu et al., 2011). Softwood lignin is predominantly composed of G units, while hardwood contains both S and G units in its lignin. There is a discrepancy on the correlation between S/G ratio and enzymatic hydrolysis efficiency. No correlation (Chen & Dixon, 2007) or strong correlation (Santos et al., 2012; Studer et al., 2011) has been reported. If there is any, the correlation would be that higher S/G ratio results in higher sugar yield. It was reported that lignin with higher hydrophilicity has less negative influence on enzymatic hydrolysis than more hydrophobic lignin (Nakagame et al., 2010). Hydrophobicity of lignin is also related to cellulase diffusion and adsorption. Beside these findings, deposition of lignin onto the surface of fibers after pretreatment was reported to adversely affect the efficiency of enzymatic conversion (Selig et al., 2007), indicating the importance of lignin distribution.

Non-productive adsorption of cellulase enzyme to lignin was proven by many research studies (Palonen et al., 2004; Sewalt et al., 1997; Yang & Wyman, 2006). This adsorption phenomenon was claimed to be spontaneous by hydrophobic, electrostatic interactions and hydrogen bonding interactions between cellulase and lignin, and the first two interactions were found to be more important than hydrogen bonding (Nakagame, 2010). Also,

hydrophobic interaction was found to be the dominating driving force of adsorption (Börjesson et al., 2007). It was suggested that heat-induced denaturation may take place for the enzyme adsorbed on the lignin surface at hydrolysis temperature (Rahikainen et al., 2011). Different strategies haven been evaluated to overcome the non-productive binding of cellulases to lignin such as surfactant addition, protein (e.g. BSA) addition, fiber treatment (e.g. alkali extraction to remove lignin with high affinity to cellulase) and lignin modification (e.g. adding carboxylic acid groups) (Eriksson et al., 2002; Nakagame et al., 2011; Pan et al., 2005; Yang & Wyman, 2006). In addition, decreasing the hydrophobicity of residual lignin after pretreatment was found to result in significant decrease in the binding of cellulases to the lignin and reducing its inhibitory effect (Eriksson et al., 2002).

Dissolved lignin was reported to reduce the activity of enzymes such as cellulases, xylanases, and glucosidases. Various cellulases differ in their inhibition by lignin. Generally, it has been shown that the xylanases and glucosidases are less affected by lignin compared to cellulases (Berlin et al., 2006).

Waste paper materials to ethanol

Among cellulosic biomass, waste paper presents a unique opportunity for bioethanol production for several reasons. First, it is a cost competitive feedstock due to the available infrastructure and networks to collect and process waste paper (Dale & Musgrove, 2009). Second, although cellulosic materials are less expensive than corn, they are more costly to convert to ethanol because of the extensive processing required associated with feedstock preparation and pretreatment to open lignocellulosic structures (DiPardo, 2004). Waste paper

biomass has gone through intensive processing such as pulping, paper making and recycling. The enzymatic amenability has already been improved since fiber swelling, cutting and cooking in the paper making process have opened the lignocellulosic structure, offering better suitability during enzymatic hydrolysis (Duff & Murray, 1996; Lynd et al., 2001). Third, converting recovered paper biomass to ethanol also has environmental benefits. It not only increases the net energy balance of ethanol compared to converting corn to ethanol (DiPardo, 2004), but also reduces the volume of landfilled MSW and it avoids generating hazardous chemicals during anaerobic digestion (Jeffries & Schartman, 1999).

In 2010, total MSW generation was 250 million tons, among which paper and paperboard products accounted for about 70 million tons (28%) of all materials in the MSW stream, Fig. 1.2 (EPA, 2010). In that same year, nearly 66 percent (or nearly 46 million tons) of all the paper consumed in the USA were recycled and this number has nearly doubled since 1990 (AF&PA, 2013). Besides recycling waste paper materials as a main or supplemented feedstock for paper production, waste paper is also considered as a potential candidate for bio-fuel production (Duff & Murray, 1996). Studies have been performed on waste paper to ethanol with different paper grades including: office paper (Chen et al., 2012; Ikeda et al., 2006; Park et al., 2002), newspaper (Kim & Chun, 2004; Kuhad et al., 2010; Lee et al., 2010a; Xin et al., 2010), carton and paperboards (Wayman et al., 1992).

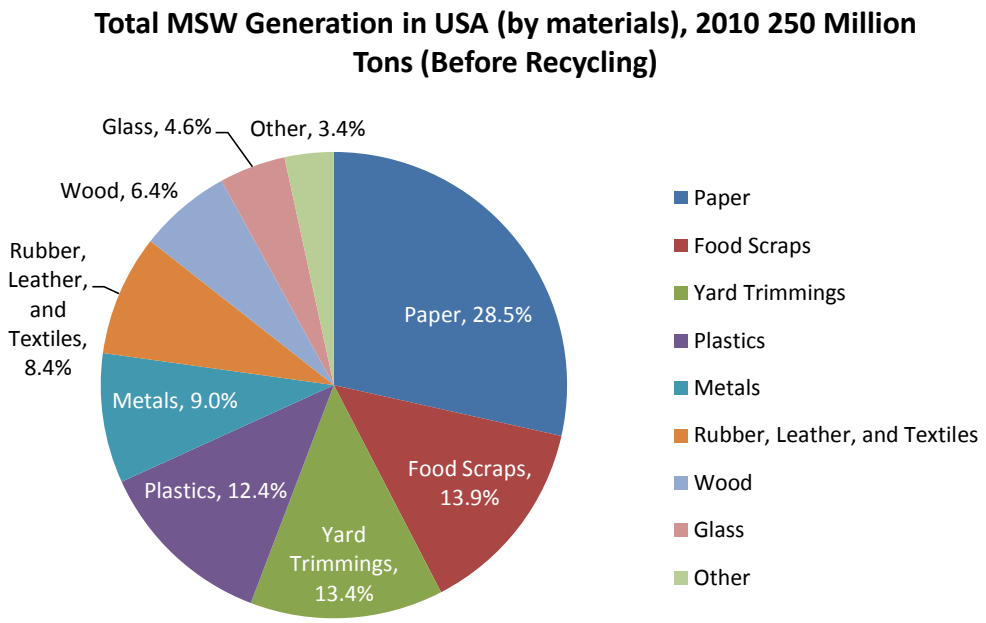

Fig. 1.2 Total municipal solid waste generation by materials in the USA in 2010

Waste paper to ethanol has been studied in terms of pretreatment techniques, enzymatic hydrolysis and fermentation conditions based on different paper grades. Generally, waste paper fiber from chemical pulps contains 60-70% cellulose, 10-20% hemicellulose and 5-10% lignin (Sun & Cheng, 2002). High enzyme dosage has mostly been used in the earlier works due to less efficient commercial cellulase from early generations and the lack of economic insights (Scott et al., 1994; Wayman et al., 1992). Due to different enzymes from various generations and suppliers and several different enzyme unit expressions, the conversion techniques from literatures are difficult to compare in terms of sugar/ethanol conversion. However, a consensus has been established that waste paper has a different nature in terms of enzymatic hydrolysis to fermentable sugar compared to woody biomass.

Office paper

Office paper has high cellulose and low lignin content since it is mostly bleached grade paper. It also contains ash in the range of 5-30% since inorganic fillers like calcium carbonate and clay are added to improve printing properties (Wayman et al., 1992). Office paper with low ash content generally requires no pretreatment (Wayman et al., 1992) or very mild acid neutralization (Shi et al., 2009) to achieve high sugar conversion. It was studied that ethanol production was increased by approximately 20% by using mixed waste office paper with ultrasound exposure for 15 min of/240 min cycle during the latter half of simultaneous saccharification and fermentation (SSF) process (Wood et al., 1997). It was believed that ultrasonic cavitation disassembled the relatively smooth surface of cellulose fibers into component microfibrils, which enabled better enzyme digestibility. In another study, a statistical method using central composite design was used to optimize enzymatic hydrolysis conditions of office waste paper (Sangkharak, 2011). In this particular case, the optimal medium composition in enzymatic hydrolysis consisted of 43.97 μg /L, 28.14 FPU/g sample and 53.73 h of wastepaper, enzyme concentration and incubation time, respectively. Wang et al. studied four types of waste paper (newspaper, office paper, cardboard and magazine) to ethanol and their financial feasibilities (Wang et al., 2013; Wang et al., 2012). By using the NREL dilute acid pretreatment process for waste paper to ethanol, it was analyzed that the cardboard-to-ethanol scenario had the lowest minimum ethanol selling price (MESP) among bioethanol produced from four types of waste paper. By modifying the NREL dilute acid pretreatment process, a MESP reduction of £0.01/l of ethanol was achieved for office paper.

Newspaper

Newsprint in the US is mainly produced from softwood kraft pulping process. It contains a substantial portion of lignin. In Wang et al.'s study (as mention above), ammonia fiber explosion (AFEX) and ammonia treatment were attempted on newspaper. It appeared that these two pretreatments did not effectively improve sugar conversion (Wang et al., 2012). However, oxidative lime using $Ca(OH)_2$ and O_2 showed a sugar yield improvement from 33% to 78% at 7.5 FPU/g cellulose (using Celluclast 1.5L). Ballesteros et al. studied fermentation of newspaper and magazine recovered paper material (Ballesteros et al., 2002). It was reported that mixing difficulties was the hurdle preventing an adequate SSF performance. Therefore, a fed-batch SSF process was performed and yielded higher ethanol production. It was also reported that semi simultaneous saccharification and fermentation (SSSF) in a fed-batch mode could generate higher concentrations of ethanol production (Elliston et al., 2013). Similar study on fed-batch fermentation of newspaper showed higher ethanol concentration than that of batch fermentation (Kuhad et al., 2010). Surfactant Tween 80 and $CoCl_2$ were also applied to improve sugar conversion in the enzymatic hydrolysis step in this study.

Recycled paper mix

Papermaking induced drying results in hornification that causes irreversible collapse of pores in fibers. In addition, recycling causes the disappearance of external fibrillation in the fibers, restoring the fine structure of the pulp to their original unbeaten state (Nazhad et al., 1995). Mechanical pretreatments such as beating and refining are often applied on recycled paper to increase accessible surface area for cellulases in enzymatic hydrolysis. One study

focused on treating recycled paper mixed with supercritical carbon dioxide. The cellulosic structure was disrupted by the explosive release of carbon dioxide and enzymatic hydrolysis efficiency was improved due to the increase of accessible surface area of fibers (Zheng et al., 1998). In another study, waste paper containing ~18% (dry basis) lignin and ~14% ash was subjected to dilute acid pretreatment. The optimum condition (0.50 N H_2SO_4 at 120 ℃ for 2 h at biomass:acid ratio of 1:10 (w/v)) resulted in 77.5% fermentation efficiency(Dubey et al., 2012).

Paper sludge to ethanol

Paper sludge is a type of solid waste material composed of shorted pulp fibers and a fraction of the paper making feedstock removed along with clays and fillers during pulping and the paper making process (Jeffries & Schartman, 1999; Lynd et al., 2001). There are two main kinds of sludges; primary sludge and secondary sludge. Primary sludge is the primary treatment residue captured through gravity settling in a primary clarifier, and it consists of fibers, fines, contaminants and fillers lost due to incomplete or inefficient solid/liquid separations at various stages of pulp and paper production. Primary sludge is approximately 3% to 10% of total paper product for processing of virgin fiber (dry basis) and 10–50% of total product for processing recycled fiber (Lynd et al., 2001; Mahmood & Elliott, 2006). Secondary sludge is a by-product of biological treatment of wastewater, and it is much more difficult to dewater than primary sludge.

Sludge disposal accounts for ~60% of the total wastewater treatment plant operating costs at pulp and paper mills (Canales et al., 1994; Cooper & Erickson, 1996; NCASI, 1992).

More than 50% of the sludge is subjected to landfilling (Lynd et al., 2001). Other options include land spreading, incineration and recycling for usage like fertilizer and animal bedding. Sludge is usually dewatered to 20-35% solids at pulp and paper mills. Landfilling is not feasible if municipalities are not designed to accept wet sludge. Transportation cost of wet sludge is high if mills are not located close to municipal solid waste disposal areas. In addition, once sludge is placed in a landfill, anaerobic digestion takes place and generates excessive acid and seepage of organic materials into the soil or as run-off (Jeffries & Schartman, 1999). Incineration of sludge is limited due to high moisture and ash content.

Like waste paper, primary sludge is a potential feedstock for ethanol production with several advantages: i) paper sludge is produced at a concentrated site with a permanent production location, making the source of sludge reliable at practically no cost (Peng & Chen, 2011); ii) the utilization of sludge for ethanol diverts material going to landfills (avoiding truck hauling costs and landfill investments) and iii) the conversion of paper sludge avoids costly pretreatment to open up the lignocellulosic structure or remove lignin to make it more amenable for enzymatic hydrolysis. Industrial paper sludge has already been subjected to extensive mechanical and chemical processes such as pulping to liberate fibers from wood, lignin removal, refining and bleaching. Compared to raw woody biomass, fibers in paper sludge are very fine and more amenable to enzymatic hydrolysis (Keating et al., 2006; Lynd et al., 2001; Wingren et al., 2003).

As of 2012, according to FisherSolve™, the primary sludge production in North America is approximately 17,300 short ton per day. In North Carolina, the total pulp and

paper production at major manufacturing centers is 2,900,000 air dry short ton (adst)/yr, Table 1.1.

If 80 gallons of ethanol per ton of sludge is produced with a value of $2.50 per gallon, the sludge available in North Carolina can yield an estimated 12 million gallons of ethanol per year with $30 million in revenue per year. With the data on sludge production according to mill types (Göttsching & Pakarinen, 2000), the total sludge availability from major pulp and paper mills in NC is estimated to be 150,600 OD tons per year.

Table 1.1 Summary of primary sludge availability in North Carolina

Company	Location	Production	Sludge quantity in Oven dry tons per yr
Blue Ridge Paper Products	Canton	BSWKP 220,000adst/yr BHWKP 295,000 adst/yr	20,600
Caraustar	Charlotte	Recycled board 54,000 st/yr	300
Cascades Tissue	Rockingham	Recycled tissue 55,000adst/yr	23,600
Domtar	Plymouth	BSWKP 305,000adst/yr BHWKP 197,000 adst/yr	20,100
International Paper	Riegelwood	BSWKP 417,000adst/yr BHWKP 442,000 adst/yr	34,400
Jackson Paper	Sylva	Recycled paperboard 104,000st/yr	500
Kapstone	Roanoke Rapids	UBKP 500,500 adst/yr	20,200
Laurel Hill Paper	Cordova	Recycled Tissue 46,000 st/yr	19,700
Weyerhaeuser	New Bern	Dissolv/Fluff Pulp 280,000 adst/yr	11,200
Total Pulp and Paper Production at major manufacturing centers in NC		2,900,000 adst/yr	150,600

Data on production from Lockwood Post Directory.
BSWKP: bleached softwood kraft pulp, BHWKP: bleached hardwood kraft pulp, UBKP: unbleached kraft pulp. Tissue mill: sludge is 30% of incoming material. Recycled liner board or fluting: sludge is 0.5% of incoming material. Board: sludge is 0.5% of incoming material
Sludge = Product/(1-fractional loss)-Product

Sludge compositions depend on the pulp and paper processes especially in terms of ash content, Table 1.2 (Lynd et al., 2001). Ash (clay, kaolin, $CaCO_3$, TiO_2 etc.) content ranges from 3% to 65% depending on the paper mill. Generally, recycled paper sludge contains higher ash content due to high filler content from starting materials. Carbohydrate content is not necessarily related to sludge origins. Total carbohydrate (glucan + non-glucan) from a large variety of sludges are reported to range from 14% to 74% of sludge dry mass. The major carbohydrate components in sludges are glucan, with some xylan and mannan. Galactan and arabinan are beyond the detection limit (Chen et al., 2014; Lynd et al., 2001).

Sludge composition analysis are usually conducted according to standard biomass composition analysis developed either by NREL (Sluiter et al., 2004) or a modified method derived from the TAPPI Standard Method T222 om-98 (TAPPI, 1998). The assumed lignin measured by these methods is essentially acid insoluble volatile matter in which most is lignin and some other volatile compounds, plus acid soluble lignin measured at a wavelength of 205 nm by UV-VIS spectrometer.

Table 1.2 Typical paper sludge origins, processes used in the mills and sludge compositions (Lynd et al., 2001)

Sludge	Origin		Composition		
I.D. #	Furnish[1]	Process[2]	Solids[3]	Glucan	Ash
1	V (HW, SW)	K, B	15.30%	37.38%	42.73%
2	R	D, B	30.11%	28.83%	28.33%
3	V	NPM, Paper	24.68%	38.05%	38.52%
4	V/R	D, U	42.13%	20.53%	65.17%
5	V	K, B, Paper	19.26%	57.14%	24.08%
6	V (HW)	K, B	40.81%	20.57%	51.62%
7	V (HW)	K, B	27.36%	42.82%	32.86%
8	R	NPM, Paper	31.88%	40.30%	4.96%
9	V	S, U	49.26%	40.85%	20.68%
10	R	D, B, Paper	32.94%	34.99%	21.83%
11	R	NPM, Paper	29.71%	53.10%	2.81%
12	V	NPM, Paper	30.48%	33.49%	15.98%
13	V/R	D, B	37.89%	26.80%	42.24%
14	V (HW, SW)	K, B	40.91%	47.40%	33.65%
15	R	NPM, Paper	36.09%	42.14%	12.39%
16	V	NPM, Paper	95.65%	19.47%	54.05%
17	R	SC, U,Paper	34.86%	42.29%	3.48%
18	R	D, B, Paper	29.82%	24.98%	34.41%
19	V	NPM, Paper	42.76%	27.87%	50.36%
20	V (HW)	S, B	36.00%	35.28%	29.60%
21	R	D, B, Paper	30.03%	22.86%	48.50%
22	R	K, B, Paper	24.84%	54.90%	26.40%
23	V	K, B	37.72%	41.69%	30.11%
24	R	D, B, Paper	32.60%	67.25%	7.20%
25	R	D, U, Paper	40.98%	47.63%	25.32%
26a	V	K, B, Paper	18.00%	60.66%	12.00%
b	V	K, B, Pulp	38.63%	17.99%	-
27	V (SW)	K, B, Pulp	37.53%	36.27%	14.85%
28	V (SW)	K, U, Pulp	19.92%	43.52%	29.43%
29a	V (SW)	S, B, Pulp	31.52%	68.59%	5.55%
b	V (SW)	S, B, Pulp	25.52%	53.97%	1.80%
30a	V	K, B, Pulp	16.57%	47.27%	19.90%
b	V	K, B, Paper	25.15%	56.53%	5.03%
31	R	D, U, Paper	36.33%	42.72%	34.88%
32	R	D, U, Paper	40.39%	33.08%	31.53%
33	V (SW)	K, B, Pulp	34.45%	32.10%	47.82%
34a	V (SW)	K, U, Pulp	37.16%	50.32%	13.59%
b	V (SW)	K, U, Pulp	7.85%	56.28%	8.00%
c	V (SW)	K, U, Pulp	13.20%	11.66%	53.98%
35	V	K, B, Pulp	40.34%	23.33%	57.37%
36	V (SW)	K, U, Pulp	16.91%	51.44%	2.35%
37	V	TC, B, Pulp	40.91%	27.20%	22.38%
38	R	D, B, Paper	48.00%	59.00%	-
39	R	D, B, Paper	33.00%	30.00%	-

[1]V=virgin, R = recycled, HW = hardwood, SW = softwood. [2]D = deinking, K = kraft, S = sulfite, TC = thermochemical, B = bleached, U = unbleached, NPM = no pulp mill, Pulp = pulp sludge, Paper = paper sludge. [3]As received dewatered from the mill.

Since sludge has already been subjected to an extensive mechanical and chemical processing imposed on raw paper material through pulping (during refining, bleaching and drying), polysaccharides in recycled paper sludge should be more amenable to enzymatic hydrolysis, as already noted by several authors (Kang et al., 2011; Lynd et al., 2001; Wingren et al., 2003).

Process development of sludge to ethanol has been studied using a two-step process that can be run as a separate hydrolysis and fermentation (SHF) process. However, enzymatic hydrolysis of paper sludge was inefficient due to the interference by large amounts of ash in the sludges during the enzymatic reaction (Kang et al., 2010). Since $CaCO_3$ in ash not only buffers the pH level (usually 2-3 units higher than the pH optimum of), making pH adjustment with acid a requirement if enzymatic hydrolysis is to occur, but also adsorb cellulase and makes inefficient bonding of cellulase to ash thereby decreasing enzyme digestibility.

Simultaneous saccharification and fermentation (SSF) was introduced to process sludge to ethanol without any pretreatment. SSF has been regarded as the major option because it usually results in higher overall yields and shorter residence times along with process integration achievements (Wingren et al., 2003). SHF and SSF for recycled paper sludge conversion to ethanol were implemented and compared for process efficiency in terms of product yield and production rate (Marques et al., 2008). In SSF, the extent of carbohydrate conversion into monosaccharides is slightly lower than the one obtained in the SHF process. This seems to be in disagreement with the reduction in end-product inhibition of beta-

glucosidase that occurs in the course of the SSF process as a result of the rapid fermentation of the released glucose (Cantarella et al., 2004). This observation might be explained by the difference in process temperatures. The SSF process was carried out at the temperature selected for the fermentation step, 30 ℃, whereas the hydrolysis of recycled paper sludge in the SHF was performed at 50 ℃, which corresponds to higher enzyme activity. However, SSF was shown by others to accelerate ethanol production (as judged by a higher ethanol volumetric production rate) when compared with the SHF process (Ooshima et al., 1985). Higher ethanol concentrations reduce the product recovery cost, as reported by several authors (Ballesteros et al., 2002; Fan & Lynd, 2007b; Moldes et al., 2000).

Fed-batch and (Jeffries & Schartman, 1999; Lynd et al., 2001) semi-continuous feeding (Fan & Lynd, 2007a; Fan & Lynd, 2007b; Fan et al., 2003) modes of SSF have also been studied. For the fed-batch fermentation, it was demonstrated that papermaking sludges can be converted to ethanol at 95% yield at small-scale (Lynd et al., 2001). However, low ethanol concentration and inhibition of cellobiose and ethanol is associated in this process. Semi-continuous/fed batch fermentation with periodic addition of fresh substrate was performed to overcome the limitation to high substrate concentrations imposed by mixing. Constraints were evaluated regarding feeding frequency and mixing energy requirements for paper sludge in relation to ethanol concentration and conversion (Fan & Lynd, 2007a). From an economic point of view, changing the feeding frequency entails a trade-off. As feeding frequency decreases, the cost of cellulase use gets lower, but higher costs are incurred for added capital associated with sludge holding tanks and larger agitator, as well as electricity to

drive agitation. Still, the economic benefits resulting from reduced cellulase loading without compromising cellulose conversion was greater.

In addition to SSF, simultaneous saccharification and co-fermentation (SSCF) of sludge has been modeled and analyzed (Zhang & Lynd, 2010; Zhang et al., 2009a; Zhang et al., 2009b). In SSCF, both cellulose and hemicellulose are utilized and converted to ethanol. It was reported that in both cases of utilizing genetically modified yeast Zymomonas mobilis 8b and Saccharomyces cerevisiae RWB222 under optimal conditions, a 19% higher overall conversion of paper sludge to ethanol was achieved than the non-xylose utilizing S. cerevisiae (Zhang & Lynd, 2010).

In the study of process design of paper sludge to ethanol, mineral recovery was taken into consideration (Fan & Lynd, 2007b). Up to 10% of the mineral content originating from SSF residues was supplemented in paper hand sheets preparation, and it was found that physical properties and optical properties like burst strength, brightness, opacity, and whiteness changed very little. Economic analysis was conducted and shows that paper mills can achieve positive cash flow with or without xylose utilization. In addition, in both SSF and SSCF processes, carbonic acid and other organic acids were formed to partially neutralize ash originating from sludge and stabilized the pH to 5.0, which is the optimum pH for cellulase (Kang et al., 2010).

Research objectives

Considering the large potential of recycling waste paper and paper sludge for bioethanol production, it is proposed to develop an optimum process for various waste paper materials as a candidate for ethanol conversion. In this process, several factors will be considered.

Firstly, ash content from waste paper and paper sludge interferences with enzyme digestibility. It could also potentially be utilized as paper filler and soil amendment. Therefore, a proposal of mechanically separating ash from sludge prior enzymatic hydrolysis was prompted in this study. The ideal fractionation process combined with the availability of utilities and other infrastructural elements at many mills offer a significant advantage for simplified processing for a facility located at a paper mill when compared to dedicated facilities processing other cellulosic materials. Finally, enzymatic hydrolysis may facilitate recovery and reuse of minerals (e.g., titanium dioxide) from paper sludge.

In previous studies, mineral recovery was considered after fermentation. However, no research has been done on mechanically separating ash from sludge and on comparing ethanol conversion efficiency with the absence of ash. Although SSF decreases the negative effect of ash on enzyme digestibility, the existence of ash, especially acid insoluble ash can adversely affect enzyme digestibility. In addition, evaluation of ash from sludge as a soil amendment alternative has not yet been studied. It is of interest to evaluate a simple and cost effective conversion process and its financial impact through simulation modeling and financial analysis.

As discussed previously, high lignin content in many types of waste paper is a hurdle for achieving ideal sugar yields in enzymatic hydrolysis. Therefore, in the last part of this research, a fundamental study on the effect of different lignin removal techniques were performed with the aim of revealing the underlying relationships among biomass structure, lignin characteristics and enzyme digestibility.

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Chapter 2 Enzymatic Hydrolysis of Recovered Office Printing Paper with Low Enzyme Dosages to Produce Fermentable Sugars

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Abstract

The use of recovered paper and paper manufacturing wastes are a potentially large, concentrated and convenient raw material for ethanol production via enzyme hydrolysis and fermentation. However, many previous studies in the area have investigated impractically high enzyme charges. In this research low dosages of enzymes on copy paper (CP) were investigated for the conditions of 5% consistency (w/v) and 50 ℃ for 48 h. The removal of inorganic filler (mainly calcium carbonate) by washing prior to hydrolysis led to higher sugar yields than the unwashed CP as well as CP acidified to remove the ash. Drying of the fibers (termed hornification) decreased the efficiency of enzyme hydrolysis, confirming previous results. The mechanical refining at 10% consistency in a laboratory refining mill of previously dried fibers improved the sugar recovery to similar or higher levels as never dried fibers. For de-ashed and refined copy paper, the sugar recovery was determined to be 82 and 97% with enzyme dosages of 4 and 8 FPU/g o.d. substrate.

Keywords: Office recovered paper, low enzyme dosage, sugar recovery, ash removal, mechanical refining, cellulase adsorption, hornification.

Introduction

A considerable amount of research interest pertaining to alternative energy sources has been triggered due to national security concerns. One of the most promising alternatives for the replacement of conventional fossil fuels is to utilize liquid fuels such as ethanol produced from biomass, or biomass-derived products (Ballesteros et al., 2002). Lignocellulosic materials, among various biomass materials, have great potential as raw materials for the production of bioethanol since these materials are the most abundant, generally not a food product, and are among the lowest-cost biomass sources in the world (Hu & Wen, 2008).

Recovered paper, among various other biomass materials, presents a unique opportunity for the production of bioethanol. It has been reported that the maximum ratio of paper-to-paper recycling is 65% (Ikeda et al., 2006). This is in part due to the recycling process shortening fibers, decreasing their papermaking potential and causing them to be used in lower grade products. As a rough general guide, the fibers become too short and weak to make paper after their 5th-6th time being recycled (Duff & Murray, 1996). Continued recycling of the fibers generates sludge which consists of fine lignocellulosic material as well as other inorganic material used in papermaking. Bioconverting the fibers existing in the sludge rendered unsuitable for paper products into valuable liquid fuels such as ethanol appears as an especially favorable option (Kang et al., 2010). Enzymatic hydrolysis provides an environmentally friendly means of depolymerizing cellulose and other carbohydrates at high yields, but the costs associated with this process may be unfavorable (Park et al., 2002). Anecdotally, it is believed from conference presentations and discussions with enzyme

producers, that enzyme dosages in the range of 4-5 FPU/g o.d. substrate or lower are necessary for a process to be economically attractive (i.e., enzyme cost in the range of $0.50 per gallon of ethanol). Developing effective systems at this enzyme charge is an important challenge.

Among all kinds of recovered paper materials, office paper/copy paper is generally recognized as having high cellulose and low lignin content. Generally, recovered paper from chemical pulps contains fibers with 60-70% cellulose, 10-20% hemicellulose and 5-10% lignin (Sun & Cheng, 2002). However, copy paper also contains a high ash content (in the range of 5-30%) since inorganic fillers like calcium carbonate and clay are added to improve printing properties (Wayman et al., 1992). Since high filler content may have a negative effect on sugar conversion in enzymatic hydrolysis, pretreatment is necessary to achieve high sugar conversion.

Previous studies have been performed on enzymatic hydrolysis of waste office paper (WOP) to fermentable sugars. It was reported that when enzymatically hydrolyzing office paper under 10 FPU/g substrate, 5% solid content and 45 ℃ for 24 h, a sugar conversion of 28.0% based on total carbohydrate with no pH control (final pH of hydrolysate 4.5) and 42.2% with pH control (pH 5.0) as attained (Park et al., 2001). In another study, it was reported that over 75% conversion in about 6 h with a solids content of 2-4% under 80-160 FPU/g of recovered paper was achieved (Scott et al., 1994), a very high and impractical enzyme dosage. It was also reported that 95% of the initial enzyme activity was still present after 24 h (Scott et al., 1994). In a study by Wayman and coworkers, a maximum saccharification of

shredded white office paper was 80.5% (based on total sugar) by adding 17.2 IU/g to shredded WOP at 10% solid content with 48 h time with no pretreatment (Wayman et al., 1992). Shi et al. (Shi et al., 2009) have reported that an enzyme hydrolysis pretreatment step with dilute sulfuric acid produced high sugar yields for various lignocellulosic feedstocks. A glucan-to-glucose yield above 80% for mixed paper under enzyme dosage of 10 mg/g (glucan and xylan in raw municipal solid waste, equivalent to about 7-10 FPU/g cellulose) was achieved after treatment with a 1% w/w H_2SO_4. The ash content (originating from inorganic filler in the paper) reported in that case was only 3.0%. Capek-Menard et al. reported that a 91% ethanol conversion rate based on maximum theoretical yield (504 L ethanol/ton paper) can be achieved by soaking white office paper in 0.5% H_2SO_4 and pretreating for 2 min at 170-220 ℃ in a Vapour Cracker (Capek-Ménard et al., 1992). The ash content in this case was not reported. Research on liquid hot water soaking (3 h, 25 to 250 ℃) as a pretreatment option for WOP has also been performed (Franceschin et al., 2010). A maximum of 85% of glucose conversion was achieved under high enzyme dosage with office paper containing 14% ash. Lynd and coworkers reported high ethanol yields with simultaneous saccharification and fermentation for pulp and paper making sludges with low ash content, which required low acid demands for proper pH adjustment (Lynd et al., 2001).

From these studies, high ash content has been shown to affect enzymatic hydrolysis. Given that most previous studies conducted enzymatic hydrolysis under high enzyme dosages which may overcome the effect of ash, it is deemed of interest to isolate and investigate filler effects on enzymatic hydrolysis. In this study, the enzymatic hydrolysis of

copy paper containing 12.9% by weight calcium carbonate under low enzyme dosages and the effects of acidification and mechanical ash removal on sugar recovery of enzymatic hydrolysis were investigated. In addition, the use of mechanical refining to overcome the effects of drying on enzymatic hydrolysis of recovered paper has also been demonstrated. The results indicate factors that influence the bioconversion of recovered paper materials containing high filler content. A promising technology that overcomes the recalcitrance of recovered office paper to enzyme hydrolysis is proposed.

Materials and methods

Raw materials

Never-dried bleached hardwood (NDBHW) and never-dried bleached softwood (NDBSW) kraft pulps were obtained from a southern kraft mill, and Office Depot Premium white copy paper (A4 sheets) was purchased from Office Depot. Ash content of the paper was measured by burning 2 g of oven-dried sample in a muffle furnace at 575±25 ℃ for 4 h as per the Tappi standard method, T 211om-85 (TAPPI, 1993a). Tests on reslushed copy paper solids recovered on Whatman 542 filter paper (18.5 cm diameter; Whatman Ltd, Maidstone, UK) resulted in an ash content of 12.9%. Of this ash, 77.6% was calcium carbonate, by Tappi standard method, T 211 om-93 (TAPPI, 1993b). Compositional analyses of all the treated materials were conducted using the NREL standard procedure (Sluiter et al., 2004). Three parallel tests were conducted for all samples. The concentration of sugars (glucose, xylose, galactose, arabinose and mannose) was quantified by a high-performance liquid chromatography (HPLC) system (Agilent 1200, Agilent, Santa Clara, CA, USA)

equipped with a deashing filter (Bio-Rad 125-0118, Bio-Rad, Hercules, CA, USA), a Shodex SP0810 column (8x300 mm, Showa Denko, Tokyo, Japan) and a refractive index detector. The mobile phase for the column was Milli-Q water at a flow rate of 0.5 mL/min at the temperature of 80 ℃.

Copy paper ash removal

CP was first torn into small pieces, soaked in deionized water for 2 h at 7.5% solid content, and then dispersed using a disintegrator for 5 min (15,000 revolutions). The pulp was then poured into the lowest chamber of a Bauer-McNett fractionator with a 200 mesh (74 μm) classifier board. The Bauer-McNett fractionator was turned on for 30 min by mixing and washing the pulp with fresh tap water. The pulp suspension was collected and filtered onto a Whatman 542 filter paper under house vacuum. Ash was measured as described above.

Acidification of copy paper

The pulped CP and 50 mM acetate buffer (pH 4.8) were added to a centrifuge tube and acidified by adding 98% sulfuric acid to adjust the pH to 4.8±0.2. The suspension was shaken gently and the pH of the suspension was measured after 1 hr. After acidification, enzymes were charged in the centrifuge tube for the hydrolysis reaction.

Pulp drying

NDBHW and NDBSW were first made into handsheets according to Tappi method T 205 om-88 (TAPPI, 1993c), with some modification. The basis weight of the hand sheet was 100 g/m2. The handsheets were then oven dried and freeze dried by the following methods.

Oven drying: handsheets were dried in a 105 ℃ air-convection oven for 24 h; Freeze drying: handsheets were dried in a freeze dryer (LabConco, Kansas City, MO, USA) for 24 h. The oven and freeze dried pulps were rewetted before enzymatic hydrolysis by soaking in deionized water at room temperature with 5% solid content for 2 h and filtered through a Buchner funnel under vacuum. The thickened pulps were then fluffed and stored in a cold room with sealed plastic zip-lock bags for at least 24 h prior to solid content testing for enzymatic hydrolysis.

Pulp refining

The dried materials were subjected to 5000 revolutions of refining in a PFI mill (Hamjern Maskin A/S, Hamar, Norway) using 30 g of o.d. pulp at 10% consistency, following Tappi procedure T 248 cm-85 (TAPPI, 1993d). The pulps were then filtered through a Buchner funnel with coarse filter paper under vacuum. After filtration, the pulps were fluffed, evenly divided, and sealed in plastic zip-loc bags in a cold room until needed.

Enzymes

Cellulase complex from Trichoderma reesei (NS50013), β-glucosidase from Aspergillus niger (NS50010) and hemicellulase (NS50014) preparations were kindly supplied by Novozymes A/S (Franklinton, NC, USA). The activity of the cellulase (NS50013) was determined to be 82.5 Filter Paper Unit (FPU)/mL according to methods described by Ghose (Ghose, 1987). The activities of the β-glucosidase (CBU = cellobiase unit, expressed as μmol of cellobiose that is converted into glucose per minute with cellobiose as a substrate) and

xylanase (FXU = Fungal xylanase unit, equivalent of endo-xylanase activity) were provided by the manufacturer.

Enzymatic hydrolysis

Enzymatic hydrolysis of all treated materials was carried out in 50mL plastic centrifuge tubes containing 2 g substrate (od basis) with a consistency of 5% (w/v) at 50 ℃ for 48 h and 180 rpm in an environmental incubator shaker (New Brunswick Scientific, Edison, NJ, USA). Hydrolysis experiments were performed using cellulase supplemented with β-glucosidase and hemicellulase at a ratio of 1.0(cellulase):0.3:0.3. The dry matter content of the treated solid samples and raw materials were determined by an infrared moisture analyzer (Mettler LJ16, Greifensee, Switzerland). The measurement was replicated, and the mean consistency was used in the experiment. After hydrolysis, the samples were heated in boiling water for 10 min to deactivate the hydrolytic enzyme and then centrifuged, and the supernatants were filtered through 0.45 μm HV filters for sugar analysis. Sugar concentrations of hydrolysates were determined by the HPLC system mentioned above. The sugar recovery was calculated as follows (0.9 is the correction coefficient of hydration (Iyer & Lee, 1999; Lee et al., 2010):

$$Sugar\ recovery = \frac{Sugar\ released\ (g) \times 0.9}{Carbohydrate\ content\ in\ treated\ marerial\ (g)} \times 100 \quad (1)$$

Substrate water retention value (WRV)

Water retention value (WRV) is an easily measurable parameter to quantify fiber hornification (Luo & Zhu, 2011), WRV was determined using the TAPPI Useful Method (UM256, 1981). A pad of 1400 g/m ²fiber was required in the procedure on the crucibles.

The pulp pad was centrifuged with a centrifugal force (Eppendorf North America, Hauppauge, New York, USA) of 900 rcf (2,400 rpm) for 30 min. The fiber was then removed from the crucibles and placed in a pre-weighed aluminum pan with lid, and the wet weight was recorded. The pan was then oven dried at 105 ℃ overnight. The samples were then cooled in a desiccator for 30 min before weighing the dry weight. The WRV was expressed as the weight of water one unit weight of fiber can retain, i.e.,

$$\text{WRV (g/g)} = \frac{w_{wet} - w_{dried}}{w_{dried}} \times 100 \qquad (2)$$

where

w_{wet} is the wet weight of the substrate;

w_{dried} is the oven dried weight of the substrate.

Cellulase adsorption measurements

Cellulase adsorption measurements onto bleached hardwood, calcium carbonate (Hydrocarb 90, Omya AG, Oftringen, Switzerland) and clay (KCS™, Georgia Kaolin Co., Elizabeth, NJ, USA) were separately carried out. Hydrocarb 90 is an ultrafine ground calcium carbonate for paper and board coating, and it is also commonly used as filler with a particle size of 0.70 μm and specific surface area of 12.5 m^2/g. KCS clay is coating kaolin which is utilized in the production of a variety of paper grades. A quantity of 1 g substrate (o.d. basis) was added in each centrifuge tube at a consistency of 5% to simulate the same condition of enzymatic hydrolysis. An enzyme loading of 15, 20, 25, 30, 35 FPU/g o.d. substrate of cellulase was used to investigate the adsorption and desorption of enzyme component activities since these loadings were within the detectable range of the UV–Vis

spectroscopy for both bleached hardwood and calcium carbonate. The experiments were run at 4 ℃ to avoid enzyme activity. After shaking gently, the cellulase and substrate were allowed to interact for 10 minutes, and then the mixture was centrifuged using a Biofuge 28 RS (Heraeus Sepatech, Osterode, Germany) at 5300 rpm for 10 min at 4 ℃. After centrifugation, 0.1 mL of supernatant was pipetted into a 20 mL glass scintillation vial. Supernatant protein was quantified using the Bradford protein assay by adding 3mL Bradford reagent (Sigma-Aldrich B6916, St. Louis, MO, USA) into the vial afterwards (Bradford, 1976). After allowing a reaction time of 20 min, measurements of UV–Vis absorption (Perkin Elmer, Waltham, MA, USA) at λmax 595 nm of the supernatant samples were taken to determine the unadsorbed protein content in the supernatant. Total protein was measured using bovine serum albumin as the standard, per the manufacturer's instructions. The amount of adsorbed protein in the supernatant was reported as a percentage of the amount of protein present in the substrate blank. The maximum adsorbed enzyme amount, A_{max}, was determined by Langmuir isotherms (Ooshima et al., 1990), according to the following equation:

$$\frac{1}{[A]} = \frac{1}{K_{ad}A_{max}}\frac{1}{[E]} + \frac{1}{A_{max}} \quad (3)$$

where

$[E]$ is the cellulase protein concentration at adsorption equilibrium in mg/mL;

$[A]$ is the adsorbed amount of protein on the substrate surface in mg/g;

A_{max} (mg/g) is the maximum amount of protein adsorbed on substrate surface;

K_{ad} is the equilibrium constant in mL/mg.

Results and discussion

Compositional analysis

The compositional analysis of NDBHW, NDBSW, CP and reduced ash CP are summarized in Table 2.1. The lignin content of the fully bleached hardwood and softwood pulps as well as the CP were too low to be detected with the methods herein. The ash content of the original CP was determined to be 12.9%, and of the ash, 77.6% was determined to be calcium carbonate. The Bauer-McNett fractionator washing process reduced ash content from 12.9% to 0.61% in CP. The carbohydrate contents of the CP and bleached hardwood and softwood were all similar, being close to 96% carbohydrate of the organic fraction. The efficiency of ash removal was measured three times and an average of 86.2% carbohydrate fraction of CP was recovered during the washing process.

Table 2.1 Chemical composition of NDBHW, NDBSW, CP and reduced ash CP. The average and standard error of three replicates were reported.

Substrate	Total Carb (%)	Glucan (%)	Xylan (%)	Ara+Man (%)	Ash (%)	Mass balance (%)
NDBHW	97.6	73.9±0.2	18.6±0.1	5.14±0.5	0.27±0.02	97.9
NDBSW	95.0	76.9±0.2	8.36±0.03	9.79±0.3	0.39±0.06	95.4
CP	83.9	63.4±0.2	13.5±0.3	7.05±0.8	12.9±0.1	96.8
Reduced ash CP	95.7	72.3±0.2	15.4±0.3	8.04±0.8	0.61±0.02	96.3

Note: NDBHW-never dried bleached hardwood; NDBSW-never dried bleached softwood; CP-copy paper.

Effect of ash removal on enzymatic hydrolysis

The main component of ash in CP is filler added to improve optical and surface properties, and the most commonly used filler is calcium carbonate (Subramanian et al.,

2007). The pH of the CP in this experiment was 8.5, due to the calcium carbonate in the paper. The pH values of the hydrolysates decreased from 7.8 to 5 with increased enzyme dosage for the CP, Table 2.2. Since the optimum pH value for the enzymes is 5, this may partly explain the higher sugar conversion yield at 8 FPU/g o.d. substrate.

Table 2.2 Sugar recovery and hydrolysate pH of NDBHW, NDBSW and CP under different cellulose dosages. Sugar recovery was based on total carbohydrate content and each data point is the average of two replicates. The average and range of duplicate experiments is reported.

Substrate		2 FPU/g	4 FPU/g	8 FPU/g
Never dried BHW	%	64.0±0.8	87.1±0.7	91.9±0.2
	pH	5	5	5
Never dried BSW	%	61.7±1.1	83.9±0.3	98.6±0.2
	pH	5	5	5
Copy paper	%	34.4±0.6	52.5±0.5	54.9±1.4
	pH	7.8	7	5

The effects of two pretreatments, acidification and filler removal on hydrolysis efficiency have been compared (Fig. 2.1). Copy paper with no pretreatment reached 54.9% conversion at 8 FPU/g. Acidification improved the sugar production by about 5% at 4 FPU/g of cellulase dosage and 16% at 8 FPU/g relative to no treatment by modifying the pH to 5 during hydrolysis. Compared to CP with acidification, the reduced ash CP had significantly higher sugar recovery at all enzyme dosages. Compared to NDBHW, the reduced ash CP was only lower by 6% at the highest enzyme dosage. The reduced ash CP demonstrated much better hydrolysis than the acidified CP at the same pH of 5. Ash content of copy paper was still 4.1% after pH adjustment, indicating that even with pH control the filler is interfering with the hydrolysis.

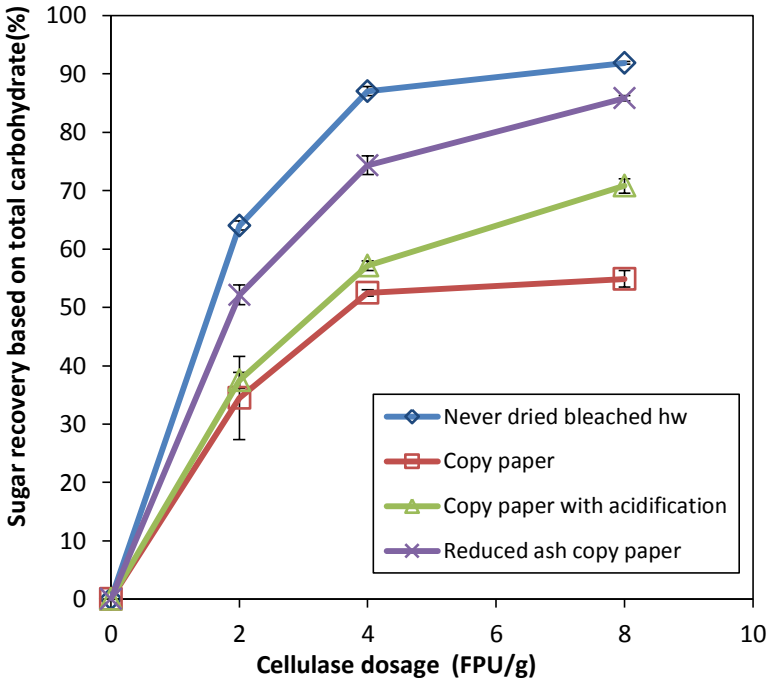

Fig. 2.1 Sugar recovery of NDBHW, CP, CP with acidification and reduced ash CP. Sugar recovery was based on total carbohydrate content and each data point is the average of two replicates; error bars depict ±1 standard error of the mean

The interference on hydrolysis from ash may be produced by direct interactions (eg., adsorption) between the enzyme and the calcium carbonate or from an indirect interaction such as the effect of the calcium carbonate on the pH of the system. The cellulase adsorption data (Fig. 2.2) indicated that for example at 15 FPU/ o.d. g substrate cellulase dosage, bleached hardwood adsorbed 93% of the total cellulase dosage while 17% cellulase adsorption was observed in a separate measurement of calcium carbonate and 38% for clay under the same cellulase dosage. Thus, an appreciable adsorption of enzyme onto the filler observed is expected to have a negative impact on the enzyme hydrolysis efficiency. This may be especially important for very low charges of enzyme.

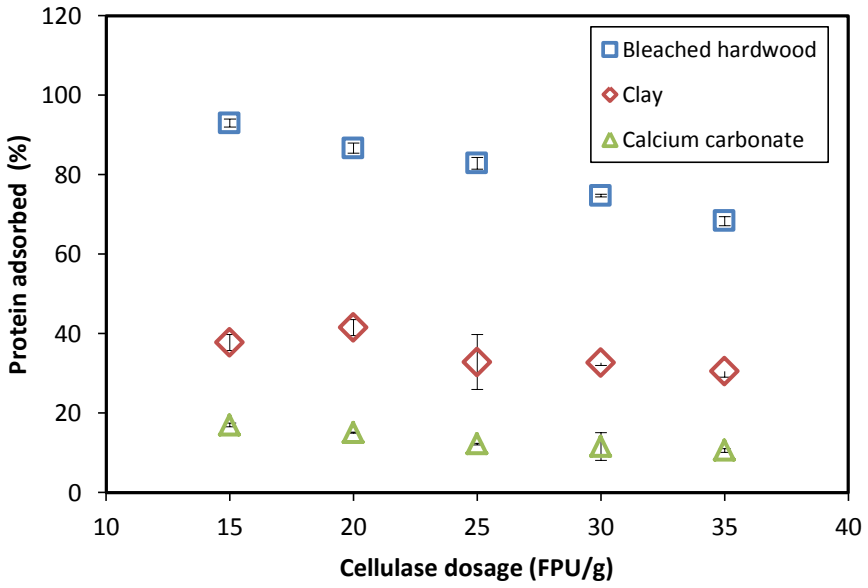

Fig. 2.2 Cellulase adsorbed as a percentage of the total cellulase added on calcium carbonate, bleached hardwood and clay (separately) under different cellulase dosages. Each data point is the average of two replicates

In addition, a Langmuir adsorption equation was applied to the results of adsorption of enzymes to single components in paper to determine the adsorption equilibrium constant K_{ad} and the maximum adsorption amount A_{max} in mg protein/ o.d. g substrate (Fig. 2.3 and Table 2.3). A_{max} of bleached hardwood, clay and $CaCO_3$ are 14.8, 12.2 and 2.71 mg/g respectively, reflecting a higher capacity of the hardwood fibers to adsorb the cellulase. The K_{ad} parameter indicates the affinity of the adsorbed material to the substrate (Ooshima et al., 1990). The K_{ad} values of hardwood, clay and $CaCO_3$ are 0.058, 1.322 and 2.902 mL/mg, indicating the affinity of cellulase for bleached hardwood was much lower than that for calcium carbonate or clay, suggesting that in an enzyme limited system, that the clay and $CaCO_3$ would compete and consume enzyme in the presence of fibers. The indirect effect of

pH may also play a role in the copy paper enzyme hydrolysis. The enzyme activity is optimal at a pH of 5. It is observed that the hydrolysate pH is much higher for the copy paper at low FPU/g values, possibly impacting the sugar recovery (Tables 2.2).

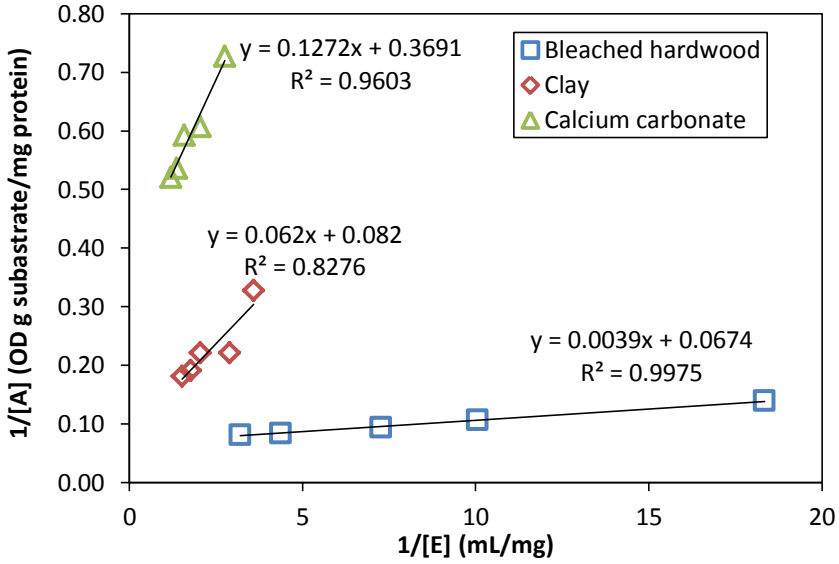

Fig. 2.3 Cellulase adsorption on calcium carbonate, bleached hardwood and clay in the Langmuir equation

Table 2.3 Equilibrium constant K_{ad} and the maximum adsorption mount A_{max} of calcium carbonate, bleached hardwood and clay

Substrate	A_{max} (mg protein/ o.d. g substrate)	K_{ad} (mL/mg)
Bleached hardwood	14.8	0.058
Clay	12.2	1.322
Calcium carbonate	2.71	2.902

Drying effect on enzymatic hydrolysis efficiency

Hornification is the irreversible fiber wall pore closure that occurs during drying when interfibrillar water of never dried fibers are removed increasing the extent of cross-linking

between adjacent microfibrils. These crosslinks are due to interfibrillar hydrogen bonds and are considered irreversible structural changes of fibers (Luo & Zhu, 2011; Weise, 1998). Hornification changes the stiffness, water absorptivity, chemical reactivity, and brittleness of the fibers. Drying causes hornification of the fines of chemical pulps (lignin free) but not of the fines of mechanical pulps (Laivins & Scallan, 1996). Consequently, when enzymatically hydrolyzing bleached pulps, the collapse of the microfibrils pore structure reduces the accessible surface area of the fibers which reduces enzyme accessibility to cellulose (Jeoh et al., 2007).

For both bleached hardwood and softwood, oven drying in general decreased the sugar production significantly (Fig. 2.4). It was also observed that oven drying had a more negative effect on sugar production than freeze drying, as calculated by the reduction in sugar recovery. This is due to the fact that freeze drying preserves more pores within a fiber structure than does normal drying, and thus avoids the hornification process to a large extent (Esteghlalian et al., 2001). At 4 FPU/g, the difference between dried and never dried fibers was the most significant. The reduction on drying is similar to previous research conducted at higher enzyme dosages (40 FPU of Celluclast/g dry pulp) and longer times (72 h) (Esteghlalian et al., 2001). In that study, a conversion rate of 98% for never dried kraft pulp and 77% for the dried pulp were reported.

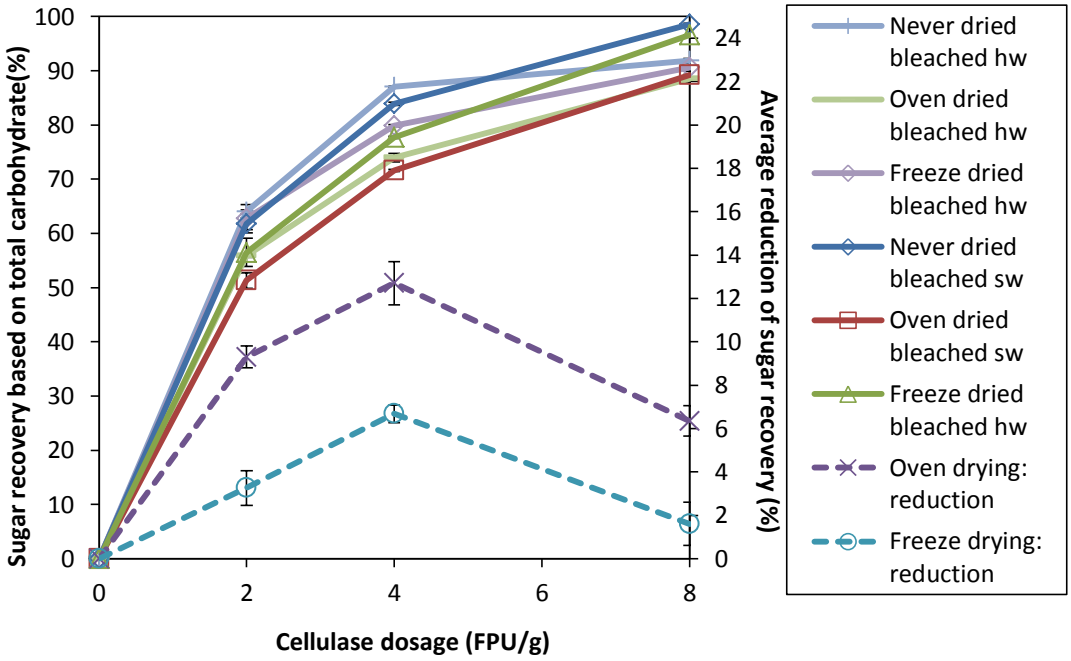

Fig. 2.4 Sugar recovery and sugar recovery reduction of never dried and dried bleached hardwood and softwood. Each data point is the average of two replicates; error bars depict ± 1 standard error of the mean

There is an apparent peak in reduction of sugar recovery for both the oven and freeze drying. This may be explained by limitations in the expected enzyme activity at very low enzyme loading of 2 FPU/g and by the high enzyme dosage of 8 FPU/g overcoming the hornification effect. The chemical composition, thermal/moisture history, and properties of oven dried bleached hardwood and reduced ash CP were similar. It was determined that under the same cellulase dosage, oven dried bleached hardwood and reduced ash CP reached similar sugar conversion rate, again confirming that the removal of ash has significant benefit. The conversions of these two types of hornified fibers were significantly less than NDBHW.

Effect of refining on sugar conversion efficiency

As mentioned previously, drying of cellulosic fibers is a mainly irreversible process, i.e., simple rewetting does not fully restore the properties. The hornification during drying can decrease the sugar production up to about 13% relative to never dried fibers (Fig. 2.4). By using a PFI mill, a very low intensity and high energy refining device (Kerekes, 2005), the swelling of fibers and fines may be restored to their never-dried levels, reflective of increased surface area, with only a modest increase in fines (Laivins & Scallan, 1996). Refining of fibers resulted in considerably higher surface areas, which has been reported as a determinant of the rate and degree of hydrolysis of the substrate (Nazhad et al., 1995).

Refining the never dried fibers (5k revolutions with PFI mill) increased the sugar recovery for both hardwood and softwood (Table 2.4). This is presumably due to fiber cutting, fibrillation, and intra-fibrillar delamination of the fibers which increases the accessible surface area for the enzymes. It was of interest to determine if the same mechanical action could overcome the effect of drying and hornification. For the bleached hardwood and softwood fibers that had been either oven or freeze dried, a 5k revolution refining treatment using a PFI mill was able to return the sugar recovery to levels the same as or better than the corresponding never dried fibers. As to be expected, the freeze dried and then refined hardwood and softwood had the best performance among dried pulps in terms of sugar conversion.

Table 2.4 Sugar recovery and average increase of sugar recovery of refined hardwood and softwood with different drying methods. The average and range of duplicate experiments is reported.

Wood type	Treatment	FPU/g	Without refining (%)	With refining (%)	Avg. % increase
Hardwood	Never dried	2	64.0±0.8	72.6±0.1	8.60%
		4	87.1±0.7	87.9±0.1	0.80%
		8	91.9±0.2	92.2±0.3	0.30%
	Oven dried	2	55.9±0.3	67.9±0.2	12.00%
		4	74.0±0.8	83.7±0.4	9.80%
		8	88.5±0.8	94.6±0.1	6.10%
	Freeze dried	2	62.8±2.6	74.4±0.6	11.60%
		4	79.9±0.2	88.6±0.3	8.70%
		8	90.6±0.03	92.3±0.1	1.70%
Softwood	Never dried	2	61.7±1.1	64.8±0.1	3.10%
		4	83.9±0.3	85.3±0.4	1.40%
		8	98.6±0.2	98.1±0.8	-0.50%
	Oven dried	2	51.3±1.4	59.4±0.4	8.10%
		4	71.6±0.2	79.3±0.3	7.70%
		8	89.2±0.7	96.2±0.2	7.00%
	Freeze dried	2	56.5±0.1	62.4±0.4	5.90%
		4	77.7±0.4	82.2±0.1	4.50%
		8	96.6±0.1	97.3±0.	0.70%

Regarding the average increase of sugar recovery for dried and refined hardwood and softwood fibers relative to dried and unrefined fibers (Table 2.4), the general trend was that the highest average increase occurred at the lowest enzyme dosage, 2 FPU/g. At 2 FPU/g an average increase of around 10% was achieved with refining on dried fibers. With increased enzyme dosage, the average % increase upon refining decreased. The decreasing average % increase of refining never dried fibers with increased FPU/g was also observed. The never

dried hardwood and softwood at the highest enzyme dosage already achieved the maximum sugar recovery and thus little potential improvement on refining would be expected. Table 2.5 shows that refining also increases the sugar recovery of reduced ash refined CP in a similar manner as oven-dried refined HW. This result indicates that a trade-off between higher enzyme dosages and mechanical refining exists and may be exploited for economic, environmental or processing objectives.

Table 2.5 Sugar recovery and hydrolysate pH of oven dried refined bleached hardwood and reduced ash refined CP. The average and range of duplicate experiments is reported

Substrate		2 FPU/g	4 FPU/g	8 FPU/g
Never dried BHW	%	64.0±0.8	87.1±0.7	91.9±0.2
	pH	5	5	5
Oven dried refined BHW	%	67.9±0.2	83.7±0.4	94.6±0.1
	pH	5	5	5
Never dried BSW	%	61.7±1.1	83.9±0.3	98.6±0.2
	pH	5	5	5
Oven dried refined BSW	%	59.4±0.4	79.3±0.3	96.2±0.2
	pH	5	5	5
Reduced ash refined CP	%	66.1±0.5	81.7±0.4	96.6±1.6
	pH	5	5	5

Refining improved sugar recovery for the untreated CP, acidified CP and reduced ash CP (Fig. 2.5). Even with the untreated CP which had pH and filler issues in terms of enzymatic hydrolysis, refining still improved sugar recovery. Reduced ash CP with refining had a 97% sugar recovery that was significantly increased relative to without refining which yielded 86% sugar recovery. In addition, even with refining, acidified CP still could not achieve a conversion yield as high as unrefined reduced ash CP.

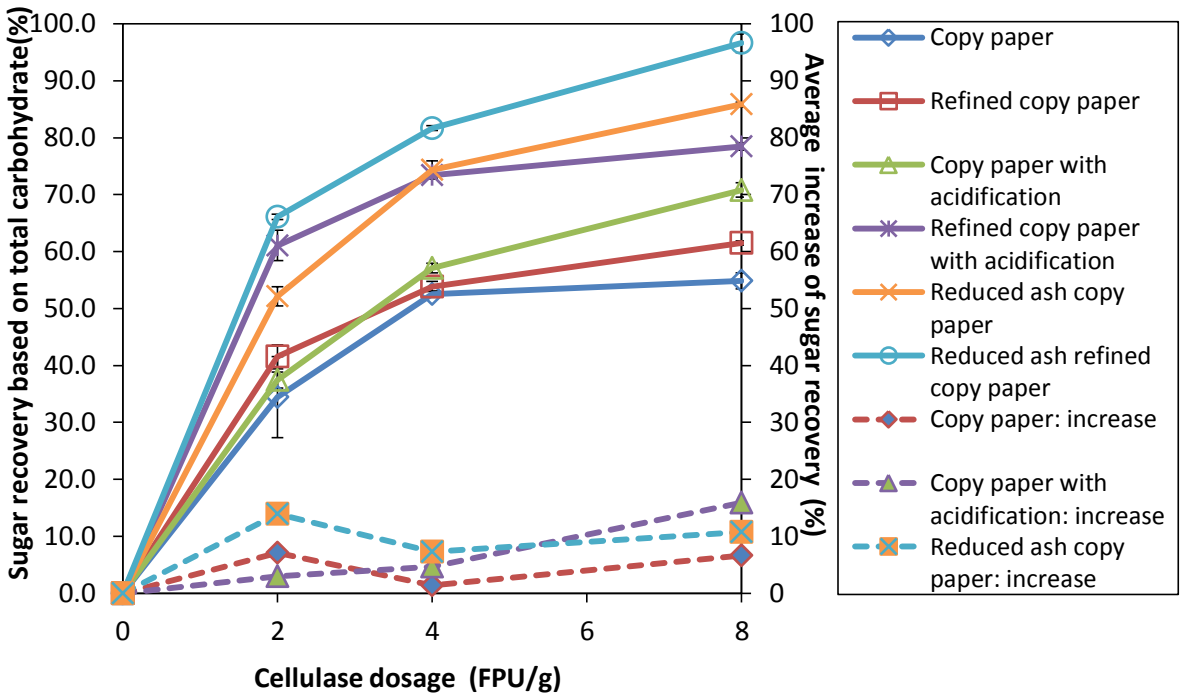

Fig. 2.5 Effect of refining on the sugar recovery of copy paper with different treatments. Also shown as filled symbols is the increase due to refining of sugar recovery versus the corresponding unrefined material. Each data point is the average of two replicates; error bars depict ± 1 standard error of the mean

Moreover, it was interesting to observe a general increasing trend of average increase of sugar recovery with enzyme dosage on acidified CP. This could be explained by the mechanical refining dispersing the filler, decreasing the particle size, and potentially making it more reactive to the acid and thus removing more filler from the system.

In summary, it has been shown that recovered office paper with ash reduced by a washing step and then refined produces a material that can have sugar conversion efficiencies of 66 to 97% for 2 to 8 FPU/g enzyme charges. The overall sugar conversion efficiencies from starting copy paper to sugar is 54.9% for untreated copy paper, 70.8% for acidified

copy paper, and 74.0% for fractionated copy paper (all at 8 FPU/g). The higher overall yield for fractionated copy paper, as well as economic and technical considerations, a reduction of raw chemicals, disposal issues and environmental concerns may point to the fractionated process being desirable. An economic analysis of the process is underway.

Effect of drying and refining on WRV

The effect of drying on fiber hornification is reflected in the WRV, which mainly reflects the ability of the fiber to retain water in the pores of the cell wall. The highest WRV were refined never-dried bleached hardwood and reduced ash refined copy paper (both 3.2 g/g). Lab drying in this research was shown to decrease the WRV to lower values than for copy paper, indicating that the drying procedure was more severe than for the industrial copy paper, Fig. 2.6(a). Mechanical refining increased the WRV and softwood from 2.0 to 2.8 g/g, and refining restored the WRV of oven-dried bleached hardwood and softwood to their never- dried level or even slightly better. Increases in sugar recovery of both HW and SW were correlated with increased WRV, for samples with or without drying and refining, Fig. 2.6(b) and (c).

Generally, these correlations are stronger and have higher slope at low FPU than at high FPU. This again demonstrates that the use of refining and the reversal of hornification as measured by WRV is very useful at low enzyme charges. While it is acknowledged that fiber cutting may play a role in the WRV, it is noted that, by comparing never-dried hardwood (or softwood) to its oven-dried state, it is observed that sugar recovery decreases even though the fiber lengths and fines content are not changed. Thus, increased sugar recovery via refining

as measured by WRV is associated with the dehornification of the fiber which provides a more accessible matrix for enzyme hydrolysis.

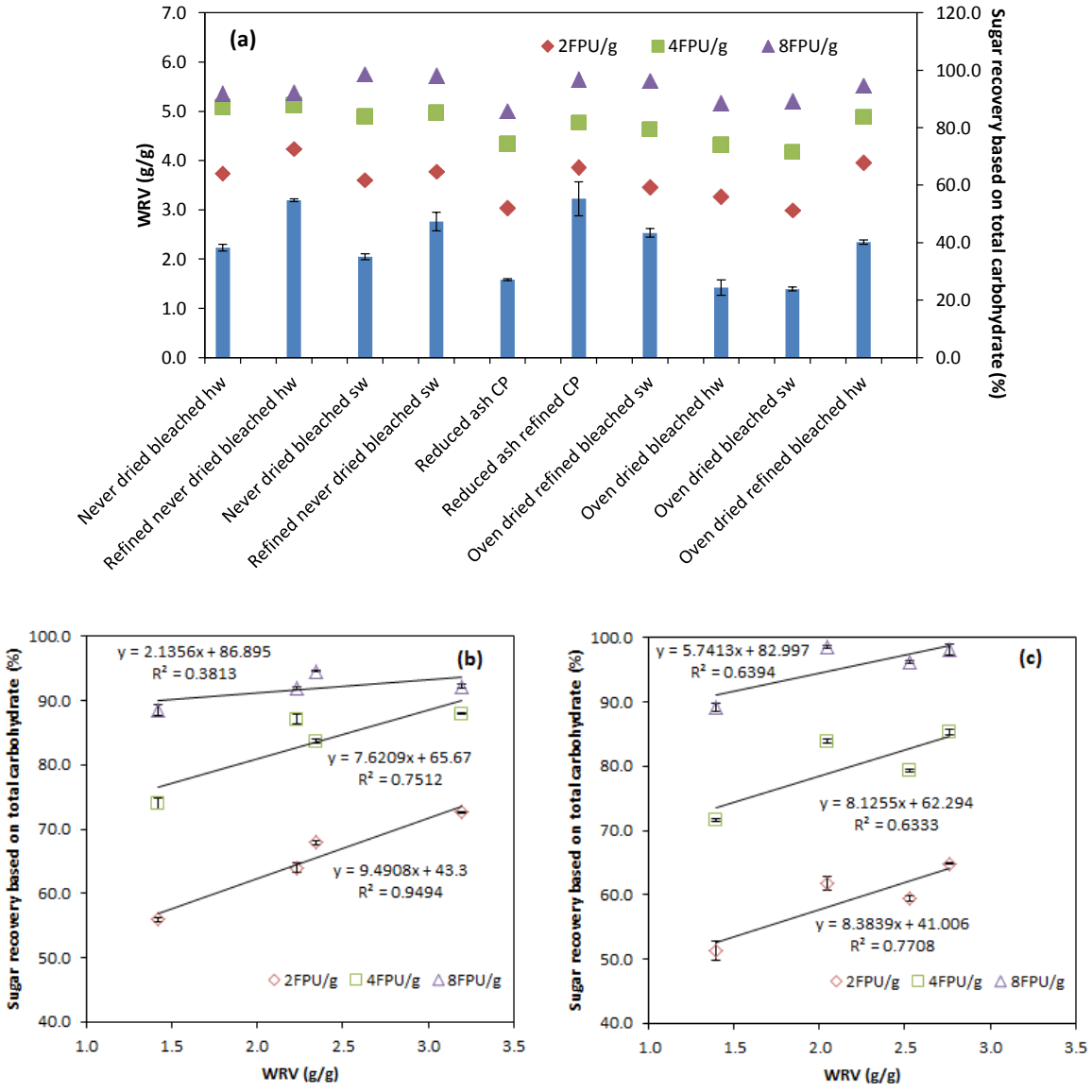

Fig. 2.6 Effect of drying and refining on WRV: (a) WRV of several low or ash free fibers with their corresponding sugar recovery based on total carbohydrate in each substrate; (b) sugar recovery versus WRV sugar of never-dried, oven-dried, refined, oven-dried refined hardwood; (c) sugar recovery versus WRV of never-dried, oven-dried, refined, oven-dried refined softwood. Each data point is the average of two replicates; error bars depict ±1 standard error of the mean

Proposed Process Flowsheet

A simplified process flow diagram for the conversion of fractionated copy paper to ethanol is shown in Fig. 2.7. The raw material might consist of a recovered mixed paper grade, but should predominantly include lignin free paper as present in most recovered office papers. There is significant potential for papermaking sludges to also be used, considering that they are often rich in fiber material and are generally very inexpensive relative to recovered paper. Other sources of carbohydrate, such as municipal solid waste, might also be used to augment the feedstock. The pulping operation would consist of high consistency pulping (circa 15-20%) without chemical addition. This step would be followed by coarse screens to remove large debris such as plastics, staples, glass and other contraries at circa 1-3% consistency. At approximately 1% feed consistency, an industrial side-hill screen with mesh size of about 200 (74 μm) could be utilized to remove ash (Shammas et al., 2010).

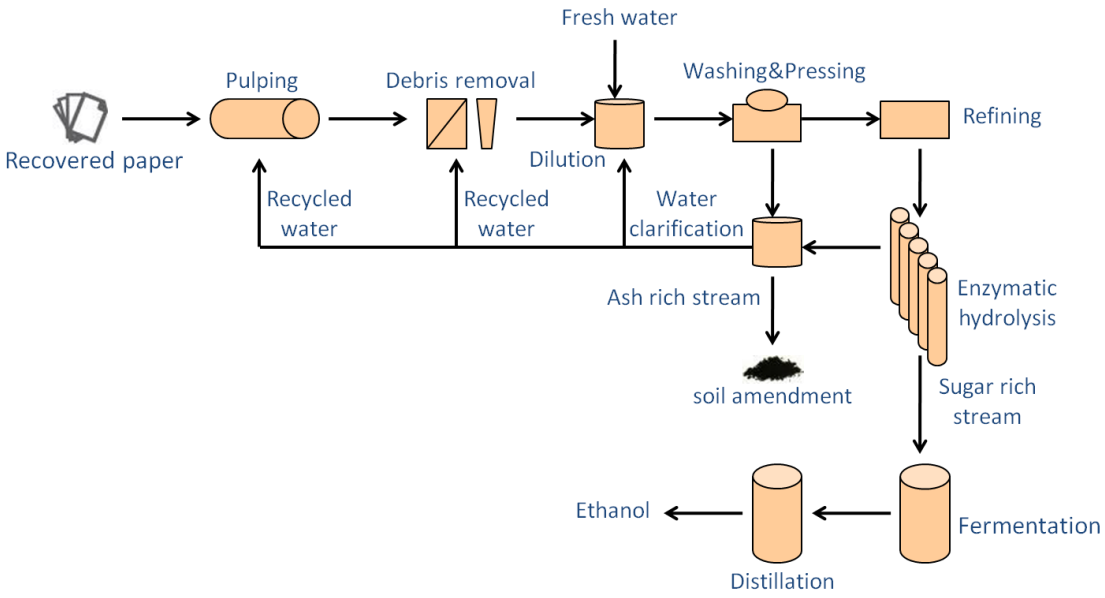

Fig. 2.7 Processing scheme to convert recovered office paper to fermentable sugars

Research is required and ongoing to determine the optimum opening size of the side hill screen and understand factors that cause carbohydrate material to be retained. A press would then be used to raise the consistency to approximately 5-12% consistency for refining (double disk refiners) and subsequent enzyme hydrolysis. The filtrates of the side hill screen and the press would be clarified using simple sedimentation or more complicated dissolved air flotation with or without flocculation chemicals. The clarified filtrate would be sent to the pulping, debris removal and dilution operations. The solids from the clarified filtrate have potential for being a valuable byproduct of the process, useful in soil amendment. Unlike refining fibers for papermaking, in this case the cutting and disintegration of the fibers does not need to be avoided and perhaps would improve subsequent hydrolysis. Further, the practical maximum consistency may be raised based on a reduction of suspension viscosity upon fiber shortening in refining. A maximum consistency in hydrolysis is preferred since upon ethanol production, the distillation operating costs decrease dramatically with increased ethanol concentration.

Conclusions

Ash interferes with the enzyme hydrolysis of lignin free paper, and absorbs enzymes with a greater affinity than fibers, decreasing the hydrolysis efficiency. A simple de-ashing followed by refining results in a 97% sugar recovery of copy paper at 8 FPU/g. Hornification, the irreversible closure of pores in fibers on drying also decreases enzymatic hydrolysis. A simple mechanical treatment of dried fibers significantly improves the fiber enzyme

digestibility. WRV is more useful to predict digestibility at low enzyme charges reflecting the use of refining and the reversal of hornification.

Acknowledgments

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Chapter 3 Conversion of Industrial Paper Sludge to Ethanol: Fractionation of Sludge and Its Impact

This chapter was accepted by *Applied Biochemistry and Biotechnology*.

Abstract

Paper sludge is an attractive biomass source for the conversion to ethanol due to its low cost and the lack of severe pretreatment required. Four sludges from pulp and paper operations including both virgin kraft (VK) and recycled & deinking (RD) paper mills were analyzed. A fractionation process using a laboratory screen was utilized to produce a fiber rich stream for enzymatic hydrolysis. This process removed 82-98% of the ash with fiber yields from 39-69%. Even though sludges in both non-fractionated and fractionated scenarios were pH adjusted, total sugar conversion was still improved by 12-27% by fractionation with 4.5 times less acid required for pH adjustment. Fermentation of the fractionated sludges showed very high ethanol yields. Acid insoluble clay adsorbs 3-5 mg enzyme per g clay depending on enzyme dosage. Acid soluble $CaCO_3$ adsorbs about half of the enzyme compared to clay. Fractionation efficiency was also evaluated by testing different size mesh screen openings (100 to 500 mesh). The 400 mesh screen presented the best fiber yield, ash removal and ash fractionation ratio for both VK and RD sludges. The ash rich streams have a lower C:N ratio than the original sludge which improves its suitability as a soil amendment.

Keywords: Paper sludge, mechanical fractionation, enzymatic hydrolysis, fermentation, enzyme adsorption, soil amendment.

Introduction

One of the most promising alternatives for the replacement of conventional fossil fuels is to utilize liquid fuels such as ethanol produced from biomass, or biomass-derived products (Ballesteros et al., 2002). Lignocellulosic materials, among various biomass materials, have great potential as raw materials for the production of bioethanol since these materials are the most abundant, generally not a food product, and are among the lowest-cost biomass sources in the world (Hu & Wen, 2008). Paper sludge is solid waste material composed of short pulp fibers, contaminants and other papermaking components such as clays and fillers (Jeffries & Schartman, 1999; McGovern et al., 1983). There are two main kinds of sludges-primary sludge and secondary sludge. Primary sludge is the primary treatment residue captured through gravity settling in the primary clarifier, and it consists of fibers, fines, contaminants and fillers lost due to incomplete or inefficient solid/liquid separations at various stages of pulp and paper production. Primary sludge is about 3% to 4% of total paper product for processing of virgin fiber (dry basis) and 15–30% of total product for processing recycled fiber (Mahmood & Elliott, 2006). Secondary sludge is a by-product of biological treatment of wastewater, and it is much more difficult to dewater than primary sludge.

Sludge disposal is viewed as a substantial disposal problem for the paper industry and represents a significant cost (around 60% of the total wastewater treatment plant operating costs) at many mills (Canales et al., 1994; Cooper & Erickson, 1996; NCASI, 1992). Paper sludge is generally subjected to landfilling, land spreading or incineration, but it still causes substantial financial cost and various environmental problems. Landfilling is not feasible if

municipalities are not designed to accept wet sludge. Transportation cost of wet sludge is high if recycle mills are not located close to municipal solid waste disposal areas. In addition, once sludge is placed in a landfill, anaerobic digestion takes place and generates excessive acid and seepage of organic materials into the soil or run-off (Jeffries & Schartman, 1999) and emit greenhouse gasses. Incineration of sludge is limited by the high moisture and ash content.

As a potential candidate raw material for producing bioethanol, paper sludge has certain advantages over other feedstocks such as agricultural residues or wood sources: i) paper sludge is produced at a concentrated site and permanent production location, making the sourcing of sludge reliable at practically no cost (Peng & Chen, 2011); ii) the utilization of sludge for ethanol diverts material going to landfill (avoiding truck hauling costs and landfill investments) and iii) the conversion of paper sludge avoids costly pretreatment to open up the lignocellulosic structure or remove lignin to make it more amenable for enzymatic hydrolysis. Industrial paper sludge has already been subjected to extensive mechanical and chemical processes such as pulping to liberate fibers from wood, lignin removal at times, refining and bleaching. Compared to raw woody biomass, fibers in paper sludge are very fine and more amenable to enzymatic hydrolysis, (Keating et al., 2006; Lynd et al., 2001; Wingren et al., 2003).

One challenge of converting paper sludge into ethanol is its high ash content and impurities (Kang et al., 2010). Our previous work (Chen et al., 2012) showed that acid soluble ash like $CaCO_3$ not only buffers the pH level (usually 2-3 units higher than the

optimum pH), but also adsorbs cellulase with a higher affinity than cellulosic fiber. Acid insoluble ash like clay also presents inactive binding with cellulase thereby decreasing enzyme digestibility of fiber in sludge. Due to the interference of large amount of ash in the sludges, enzymatic hydrolysis of paper sludge has been shown to be inefficient in separate hydrolysis and fermentation (SHF) (Kang et al., 2011; Nikolov et al., 2000).

In one study (Kang et al., 2011), the fractionation of sludge using a 100 mesh screen and air floatation showed a 10% improvement in ethanol yields at enzyme dosage of 10 FPU/g glucan in a simultaneous saccharification and co-fermentation (SSCF) process. Our previous study also showed that by fractionating recycled copy paper, enzyme hydrolysis of the fractionated material with 0.6% ash content produced approximately 40% higher sugar than unfractionated material (pH adjusted) at 4FPU/OD (oven dry) g of substrate enzyme dosage (Chen et al., 2012). Therefore, in order to achieve higher efficiency in enzymatic hydrolysis (lower enzyme dosage and higher sugar output), a mechanical fractionation approach prior to enzymatic hydrolysis was further investigated in this study.

In this research, four sludges from different mills producing either virgin or recycled paper products were evaluated. Fractionation techniques were developed to improve the overall hydrolysis efficiency of the sludges. Both non-fractionated and fractionated scenarios with different enzyme dosage profiles were studied. Fractionation could produce an equivalent yield of sugar relative to unfractionated sludge but with several other benefits that improve the economics (Chen et al., 2014).

Materials and Methods

Feedstock

Four types of primary sludge were collected from four paper mills in North and South Carolina during the summer of 2011 at approximately 50% moisture content and stored in sealed buckets in a cold room. Sludges from virgin wood kraft pulping processes were named VK and sludges from recycled deinking paper making processes were named RD. All sludges were well mixed, fluffed and stored in separate sealed plastic zip-lock bags for lab experiments. Composition analyses of all paper sludges were conducted using the NREL standard procedure (Sluiter et al., 2004). Three parallel tests were conducted for all samples. Ash content of sludge was measured using Tappi standard method, T 211om-85 with a furnace temperature of 575 ℃ (TAPPI, 1993a) and $CaCO_3$ content was measured per Tappi standard method, T 211 om-93 utilizing furnace temperatures of 575 ℃ and 950 ℃ (TAPPI, 1993b).

Enzymatic hydrolysis

Enzymatic hydrolysis was conducted at a total solids content of 5% (w/v) at 50 ℃ for 48 h and 180 rpm in an environmental incubator shaker (New Brunswick Scientific, Edison, NJ, USA). Enzyme complex - Cellic® CTec2 (cellulase complex blended with high levels of cellulases, beta-glucosidase, and hemicellulase) and Cellic® HTec2 (endoxylanase with cellulase background) were provided by Novozymes (Franklinton, NC, USA). Cellulase activity of CTec2 was determined to be 136 Filter Paper Unit (FPU)/mL using the filter paper assay as described in the International Union of Pure and Applied Chemistry standard

method (Ghose, 1987). Enzyme dosages used in this study were 2, 4 and 8 FPU/OD (oven dry) g sludge and the mixing ratio of CTec2 and HTec2 was 9:1 by volume. The pH of enzymatic hydrolysis was controlled at 4.8 by 50 mM sodium citrate buffer. For pH adjusted samples, in addition to buffer, 98% sulfuric acid was added to adjust the pH to 4.8±0.2. Sugar conversion and sugar recovery were calculated as follows.

$$\text{Sugar conversion} = \frac{Sugar\ released\ (g) \times 0.9}{Carbohydrate\ content\ in\ the\ treated\ sludge\ (g)} \times 100\%$$

$$\text{Sugar recovery} = \frac{Sugar\ released\ (g) \times 0.9}{Carbohydrate\ content\ in\ the\ untreated\ sludge\ (g)} \times 100\%$$

Fractionation

Sludge fractionation was conducted by a Pulmac MasterScreen (Pul-mac International, Montpelier, VT, USA) with a 0.2 mm hole size opening screen, Fig. 3.1.

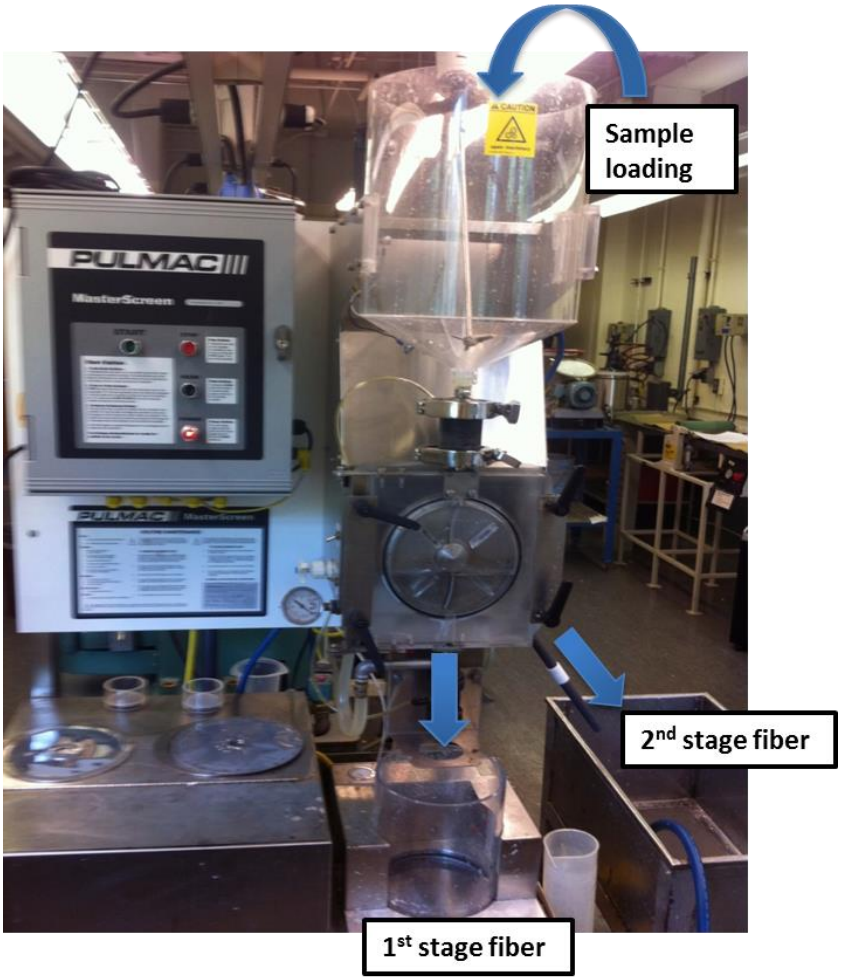

Fig. 3.1 Pulmac MasterScreen fractionation equipment

Two fiber rich streams were obtained after fractionation: (1) 1st stage fiber that would not pass through the 0.2 mm hole screen and were retained on the tray and (2) 2nd stage fiber that passed through the 0.2 mm hole screen but was retained on a 200 mesh (0.074 mm opening) screen.

To study the effect of screen size openings on fractionation, sludge was fractionated using a cylindrical jar with a screen on the bottom and agitator just above the screen, a Britt

Jar. For one batch of fractionation, 500 mL of disintegrated sludge slurry with 0.5% (w/v) solid content was poured into the device. The agitator was set to 750 rpm and the clamp from the bottom of the jar was removed to allow drainage through the screen. Water was added in increments of 500 mL when the water level reached 1-2 mm from the screen surface. This was repeated 9 more times for a total of 10 additional washes. After the last wash, the jar was allowed to completely drain. The fiber-rich sample was then collected from the screen. Screens of sizes 100, 200, 300, 400 and 500 mesh were applied for the experiments. Screen sizes used in this study are shown in Table 3.1.

Table 3.1 Screen sizes

Screens	**Opening size(mm)**	**Sample**
Pulmac screen	0.223	MasterScreen 1st stage fiber
100 mesh screen	0.152	Britt Jar fractionation
200 mesh screen	0.075	MasterScreen 2nd stage fiber
300 mesh screen	0.053	Britt Jar fractionation
400 mesh screen	0.037	Britt Jar fractionation
500 mesh screen	0.025	Britt Jar fractionation

Ash removal and ash fractionation ratio are defined as follows (Göttsching & Pakarinen, 2000):

$$\text{Ash removal} = \frac{M_i - M_a}{M_i} \times 100\%$$

$$\text{Ash fractionation ratio: } R = \frac{c_i - c_a}{c_i}$$

where

M_i and M_a are the total solid mass of inputs and accepts (accepts: fiber rich stream in this case);

c_i and c_a are the mass % (proportion) of ash of the inputs and accepts solids.

Ash removal reflects the actual ash by weight that has been removed during the fractionation process. Ash fractionation ratio reflects the efficiency of the fractionation process by comparing the proportions of the ash in each stream. Both measures may be important and provide different information. For instance, even if the ash removal is high, the ash fractionation ratio can be low if significant amounts of fiber and fines are lost in the rejects (ash rich stream).

Fermentation

T. saccharolyticum strain MO1442 was grown on MTC medium in a chemostat with a growth rate of 0.10 h^{-1}. This strain was engineered with deletion construction of phosphotransacetylase, acetate kinase, lactate dehydrogenase, a gene for exoploysaccharide accumulation and genes inserted for urea utilization from clostridium thermocellum. This culture was used as an inoculum into treated hydrolysate fermentation medium. MTC medium contained 1 ml/l 0.2% resazurin, 2.0 g/l potassium citrate ($C_6H_5O_7K_3$), 1.25 g/l citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$), 1.0 g/l sodium sulfate (Na_2SO_4), 1.0 g/l potassium phosphate (KH_2PO_4), 1.6 g/l sodium bicarbonate ($NaHCO_3$), 5 g/l urea, 1.0 g/l $MgCl_2 \cdot 6H_2O$, 0.2 g/l $CaCl_2 \cdot 2H_2O$, 0.1 g/l $FeCl_2 \cdot 4H_2O$, 1.0 g/l L-cysteine hydrochloride monohydrate ($C_3H_7NO_2S \cdot HCl \cdot H_2O$), 0.15 g/l L-methionine, 20 mg/l pyridoxamine dihydrochloride, 4 mg/l p-aminobenzoic acid (PABA), 2 mg/l D biotin, 2 mg/l vitamin B_{12}, 4 mg/l thiamine, and 5 g/l cellobiose.

The fractionated sludge enzymatic hydrolysates were further fermented by Mascoma Corporation, Lebanon, NH. The hydrolysates were neutralized to pH >5.8 by the addition of solid $MgCO_3$. The filter was sterilized and made anaerobic using vacuum and pure N_2 gas. For minimal defined medium experiments, one volume of each hydrolysate was dispensed into a sterile tube along with 0.1 volume of chemostat culture and incubated at 51 ℃ for 68 h. For medium with complex nutrients, 20 volumes of hydrolysate was mixed with the following on a per liter basis: 8.5 g BD Difco low dust yeast extract, trisodium citrate $\cdot 2H_2O$, 1g KH_2PO_4 5g urea, 0.2 g ferrous sulfate $\cdot 7H_2O$, 0.12g L-methionine, 0.5 g cysteine HCl $\cdot H_2O$, $CaCl_2 \cdot 2H_2O$ and 50% concentration of the above MTC vitamins. These bottles were inoculated with 0.05 volumes of chemostat culture and incubated at 51 ℃ for 68 h. Cultures were performed in duplicate and the averages reported.

Cellulase adsorption measurements

Cellulase adsorption was measured using cellulase complex from Trichoderma reesei (NS50013, Novozymes A/S). Substrates included bleached hardwood (BHW) fibers, $CaCO_3$, clay and $CaSO_4$. A quantity of 1 g substrate (o.d. basis) was added in each centrifuge tube at a consistency of 5% to simulate the same conditions as enzymatic hydrolysis. For the case of mixing fiber with ash, a total of 1 g of fiber and 0.5 grams of ash was dosed. A cellulase enzyme loading of 15, 20, 25, 30, 35 FPU/g o.d. substrate was used to investigate the adsorption and desorption of enzyme since these loadings were within the detectable range of UV-Vis spectroscopy. Corresponding initial enzyme concentrations were calculated in the results. The experiments were run at 4 ℃ to avoid enzyme activity. After shaking gently, the

cellulase and substrate were allowed to interact for 10 minutes, and then the mixture was centrifuged using a Biofuge 28 RS (Heraeus Sepatech, Osterode, Germany) at 5300 rpm for 10 min at 4 ℃. After centrifugation, 0.1 mL of supernatant was pipetted into a 20 mL glass scintillation vial. Supernatant protein was quantified using the Bradford protein assay by adding 3mL Bradford reagent (Sigma-Aldrich B6916, St. Louis, MO, USA) into the vial afterwards (Bradford, 1976). Measurements of UV-Vis absorption (Perkin Elmer, Waltham, MA, USA) at a λ_{max} of 595 nm was carried out after a 20 min reaction. The enzyme adsorbed on the substrate was back calculated from the measurement of free enzymes in the supernatant.

Results and discussion

Effect of sludge type and pH on enzymatic hydrolysis

Initial evaluation of VK and RD sludges without fractionation were conducted to provide a baseline for subsequent fractionation experiments. Both sludges presented alkalinity with a pH of ~8. Acidification prior to enzymatic hydrolysis was applied by incubating the sludge and buffer with sulfuric acid to achieve a pH of 5 which is the optimum pH for the enzyme activity. The buffer alone was not able to effectively adjust the pH and still maintain the target solids consistency. A large difference was shown in terms of total sugar conversion by comparing enzymatic hydrolysis of sludge with and without pH adjustment, Fig. 3.2. At the same enzyme dosage, RD1 sludge had lower sugar conversion compared to VK1 sludge even at the same pH due to its higher ash (almost double) and

impurity content, Table 3.2. It is known that ash can consume enzymes and lower the sugar conversion under these conditions (Chen et al., 2012).

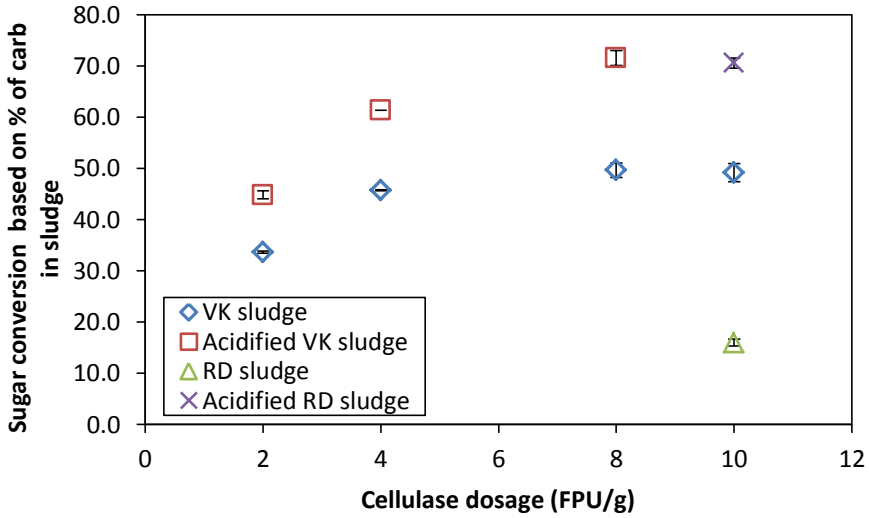

Fig. 3.2 Sugar conversion during enzyme hydrolysis of VK1 and RD1 sludge with and without acidification. Acidification was used to adjust the initial pH to 5. Each data point is the average of two replicates; error bars depict ± 1 standard error of the mean

Table 3.2 Composition of original sludges, 1st stage and 2nd stage sludges fractionated from MasterScreen

Substrate	Total carb, %	Glucan, %	Xylan, %	Arabinan+ Mannan, %	Ash, %	Acid soluble lignin, %	Klason lignin, %	Extractive, %	Mass balance, %
VK1	62.2 (0.01)	50.6 (0.1)	9.0 (0.1)	2.61 (0.03)	26.2 (0.06)	0.64 (0.002)	10.3 (0.2)	1.02 (0.2)	100.3
1-VK1	81.2 (0.5)	67.0 (0.5)	10.3 (0.01)	3.94 (0.01)	6.7 (0.2)	0.78 (0.01)	8.49 (0.4)	N/D	97.2
2-VK1	80.5 (1.1)	65.0 (0.4)	12.4 (0.2)	3.02 (0.5)	16.1 (0.05)	0.64 (0.002)	6.14 (0.09)	N/D	103.4
VK2	43.6 (0.5)	35.6 (0.3)	6.6 (0.1)	1.44 (0.02)	50.8 (0.06)	0.54 (0.01)	3.96 (0.09)	1.05 (0.2)	99.9
1-VK2	94.0 (0.1)	79.2 (0.1)	11.2 (0.1)	3.61 (0.1)	3.1 (0.04)	0.53 (0.004)	2.20 (0.2)	N/D	99.8
2-VK2	80.6 (1.5)	66.2 (0.9)	12.0 (0.6)	2.39 (0.05)	16.3 (0.1)	0.69 (0.1)	2.58 (0.2)	N/D	100.1
RD1	34.8 (1.1)	27.4 (1.1)	6.0 (0.01)	1.40 (0.06)	54.5 (0.3)	0.50 (0.07)	6.72 (0.5)	2.53 (0.1)	99.0
1-RD1	89.0 (0.3)	73.9 (0.2)	10.2 (0.1)	4.93 (0.02)	3.8 (0.05)	0.57 (0.005)	7.84 (0.07)	N/D	101.2
2-RD1	43.2 (0.5)	34.3 (0.2)	7.4 (0.2)	1.50 (0.1)	45.0 (0.1)	0.47 (0.006)	8.56 (0.1)	N/D	97.2
RD2	31.6 (0.01)	25.5 (0.1)	4.7 (0.01)	1.40 (0.1)	56.1 (0.2)	0.40 (0.002)	6.74 (0.2)	2.62 (0.1)	97.5
1-RD2	90.6 (0.1)	76.4 (0.1)	10.2 (0.01)	4.06 (0.03)	1.7 (0.1)	0.61 (0.003)	6.27 (0.06)	N/D	99.2
2-RD2	84.4 (0.6)	69.2 (0.2)	12.8 (0.01)	2.42 (0.4)	8.8 (0.06)	4.86 (0.07)	0.60 (0.02)	N/D	98.7

Note: 1: 1st stage; 2: 2nd stage.

Fractionation efficiency and enzymatic hydrolysis

Four types of sludges, two from mills that made virgin paper (VK1, VK2) and two from mills that recycled and deinked paper (RD1, RD2) were processed with a two stage ash removal process utilizing 0.223 mm diameter holes in the first stage and a wire mesh screen with openings of 0.075 mm in the second stage. The composition of the original sludges, 1st stage and 2nd stage fiber rich streams are listed in Table 3.2. Generally, the ash content of all four sludges decreased significantly after fractionation. The 1st stage fiber, which did not pass through the 0.2 mm hole screen, had lower ash content than the 2nd stage fibers which passed through the 0.2 mm hole screen and were retained on a screen with 0.075 mm openings. Total carbohydrate content of fractionated sludge was increased to ~90% for the 1st stage fiber-rich streams.

The mass balance of the sludge fractionation is listed in Table 3.3, based on 100 OD g of initial sludge fed to fractionation. The 1st and 2nd stage yields in the fiber rich material were based on the corresponding original loading of each component. VK sludges had higher overall fiber recovery yields compared to RD sludges, 69.1% and 65.5% compared to 52.7% and 38.0%. This was mainly due to more long fibers in the virgin sludges than recycled sludges. Ash removal of these four sludges ranged from 81.6% to 98.2%. For both VK and RD sludges, the ash fractionation ratio was 0.67 to 0.90, indicating reasonable fractionation with the screening barriers utilized.

Table 3.3 Mass balance of sludge fractionation based on 100 g initial loading into the MasterScreen

	In	1st stage screen retained	1st stage yield, %	1st screen pass	2nd stage retained	2nd stage yield, %	2nd screen pass	Overall yield, %	Ash removal, %	Ash fractionation ratio
Virgin Kraft Sludge 1 (VK1)										
Total	100	44.5	44.5	55.5	11.3	11.3	44.2	55.8		
Ash	26.2	2.99	11.4	23.2	1.82	6.96	21.4	18.4	**81.6**	**0.67**
Fiber	73.8	41.5	56.2	32.3	9.48	12.8	22.9	**69.1**		
Virgin Kraft Sludge 2 (VK2)										
Total	100	18.9	18.9	81.1	16.6	16.6	64.5	35.5		
Ash	50.8	0.58	1.15	50.3	2.71	5.33	47.6	6.47	**93.5**	**0.82**
Fiber	49.2	18.3	37.3	30.8	13.9	28.3	17.0	**65.5**		
Recycling Deinking Sludge 1 (RD1)										
Total	100	6.30	6.30	93.7	21.4	21.4	72.3	27.7		
Ash	54.5	0.24	0.44	54.3	3.49	6.40	50.8	6.84	**93.2**	**0.75**
Fiber	45.5	6.06	13.3	39.4	17.9	39.4	21.5	**52.7**		
Recycling Deinking Sludge 2 (RD2)										
Total	100	7.80	7.80	92.2	9.90	9.90	82.3	17.7		
Ash	56.1	0.13	0.24	56.0	0.87	1.55	55.1	1.79	**98.2**	**0.90**
Fiber	43.9	7.67	17.5	36.2	9.03	20.6	27.2	**38.0**		

The fiber rich streams obtained from the fractionation were subjected to enzymatic hydrolysis. With sodium citrate buffering, the 1st stage fiber rich sample did not need additional pH adjustment due to the low ash content. In contrast, the pH of 5% (w/v) sludge suspension would not reach 5 by the addition of buffer for the unfractionated sludge or 2nd stage fiber rich samples. Sulfuric acid was used to adjust these sludges.

Both pH adjusted and non-pH adjusted samples were compared in enzymatic hydrolysis with 2, 4 and 8 FPU/ g substrate, Table 3.4. In general, pH adjustment improved sugar conversion for the sludges and improved the conversions of glucan, xylan and mannan. The sugar conversions were generally in the order of 1st stage>2nd stage>non-fractionated sludge

for the no-pH adjustment and for pH adjustment scenarios, indicating that the ash in the samples was impacting the sugar conversions negatively. In addition, based on the same enzyme dosage as FPU/g substrate, the non-fractionated scenario always had higher enzyme dosage on the basis of FPU/g glucan compared to the fractionated scenario. However, the sugar conversions were much lower, indicating lower enzymatic hydrolysis efficiencies. The results herein showing that removal of ash improves hydrolysis is in agreement with another study in which the addition of $CaCO_3$ to bleached hardwood fibers was shown to decrease the glucan digestibility (Kang et al., 2010).

Table 3.4 Sugar conversion of original sludges and corresponding fractionated sludges

Samples	Cellulase dosage (FPU/ od g substrate)	Cellulase dosage (FPU/ od g glucan)	No pH adjustment				pH adjustment			
			Total sugar conversion (%)	Glucan conversion (%)	Xylan conversion (%)	Mannan conversion (%)	Total sugar conversion (%)	Glucan conversion (%)	Xylan conversion (%)	Mannan conversion (%)
VK1	2	4.0	40.2	37.0	63.3	21.2	48.0	45.9	64.8	27.5
	4	7.9	51.4	47.9	75.4	37.7	59.7	57.4	79.4	33.6
	8	15.8	52.7	46.7	87.8	48.2	82.3	81.3	96.2	49.7
1-VK1	2	3.0	55.7	54.2	74.3	32.6	/	/	/	/
	4	6.0	80.1	79.4	95.4	52.9	/	/	/	/
	8	11.9	96.2	95.8	98.8	67.0	/	/	/	/
2-VK1	2	3.1	64.2	62.9	79.2	28.6	58.5	56.7	76.3	23.2
	4	6.2	89.0	88.7	98.3	46.9	75.1	74.0	90.5	33.9
	8	12.3	97.2	97.5	98.2	51.1	98.7	98.6	98.9	56.5
VK2	2	5.6	34.3	28.5	69.5	15.2	58.6	55.6	87.6	15.2
	4	11.2	47.8	39.9	94.5	27.6	63.4	58.0	106.3	27.6
	8	22.5	49.6	40.5	98.2	50.2	47.7	39.1	103.6	50.2
1-VK2	2	2.5	62.1	60.5	82.8	32.2	/	/	/	/
	4	5.1	91.7	91.1	97.4	59.2	/	/	/	/
	8	10.1	99.7	99.6	99.8	74.7	/	/	/	/
2-VK2	2	3.0	42.4	41.0	56.7	12.0	71.7	70.4	88.0	27.5
	4	6.0	67.8	66.4	82.8	30.7	91.3	90.3	99.7	42.1
	8	12.1	88.2	87.2	99.8	51.7	99.8	99.7	99.9	55.3
RD1	2	7.3	58.8	54.8	84.6	25.7	65.8	60.9	95.8	31.0
	4	14.6	67.0	61.9	98.2	34.4	56.4	47.8	99.7	31.8
	8	29.2	52.7	43.5	98.6	38.7	57.8	47.7	99.7	41.6
1-RD1	2	2.7	52.0	50.7	73.0	28.1	/	/	/	/
	4	5.4	71.3	70.5	90.1	44.5	/	/	/	/
	8	10.8	85.2	84.8	99.7	57.7	/	/	/	/
2-RD1	2	5.8	51.7	48.7	72.6	18.4	70.9	68.6	90.3	28.2
	4	11.7	67.0	62.7	92.9	36.1	83.5	82.4	98.3	33.9
	8	23.3	65.8	58.3	103.6	49.2	81.4	77.0	108.3	50.2
RD2	2	7.8	78.9	78.7	96.9	23.3	87.7	88.1	97.9	26.5
	4	15.7	92.3	92.6	99.1	35.1	94.7	95.6	98.5	30.8
	8	31.4	98.0	98.2	98.9	43.1	99.2	99.1	99.3	40.1
1-RD2	2	2.6	56.9	55.4	80.0	26.1	/	/	/	/
	4	5.2	74.9	73.9	95.7	41.9	/	/	/	/
	8	10.5	93.1	92.3	98.7	58.5	/	/	/	/
2-RD2	2	2.9	46.3	45.0	60.1	11.3	63.1	61.6	79.1	20.2
	4	5.8	67.4	66.5	81.1	20.9	95.8	95.7	99.2	27.2
	8	11.6	87.2	87.2	97.3	35.0	97.6	97.1	99.6	48.0

Note: 1: 1st stage; 2: 2nd

Overall sugar conversion, enzyme adsorption and fermentation trials

The overall sugar conversion based on mixing the 1st stage and 2nd stage fiber rich samples according to their yields were calculated, Fig. 3.3. This calculation assumes that the

enzyme hydrolysis extents would not be affected by mixing the 1st and 2nd stage fiber rich materials together. It is shown that by fractionation, sugar conversions were generally improved relative to the non-fractionated sludge. The improvement for virgin kraft sludges was especially significant. For example, sugar conversion of VK2 sludge was increased from 64.3% to 91.5%. For RD2, the difference between fractionation and non-fractionation was not significant due to the possibility that 4 FPU/g was an enzyme overdose for this particular sludge which overcame the negative impact of ash on the hydrolysis.

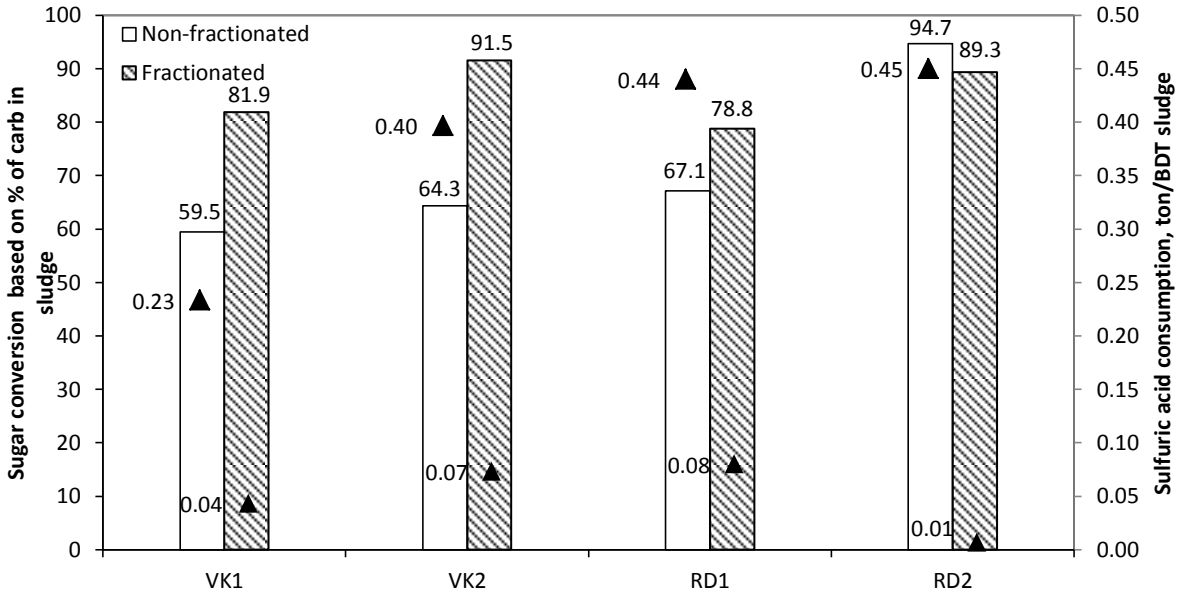

Fig. 3.3 Sugar conversions of non-fractionated and fractionated VK1, VK2, RD1 and RD2 sludges at 4 FPU/g enzyme dosage and its corresponding sulfuric acid consumption prior to enzymatic hydrolysis, ▲: sulfuric acid required to adjust the pH for enzymatic hydrolysis, ton/BDT sludge

In related research Lynd and co-workers determined that increased ethanol yields from papermaking sludges required significant amounts of pH adjustment with acids for different sludges (Lynd et al., 2001). Another advantage of fractionating sludge is the reduction of

sulfuric acid demand for adjusting the pH of the sludge slurries. For the non-fractionated scenario, the sulfuric acid requirements for VK1, VK2, RD1 and RD2 were 0.23, 0.40, 0.44 and 0.45 ton/bone dry ton (BDT) sludge; for the fractionated scenario, the sulfuric acid demand was lowered to 0.04, 0.07, 0.08 and 0.01 respectively, Fig. 3.3. By adding a fractionation operation to a sludge to ethanol process, the chemical costs are decreased significantly (Chen et al., 2014).

It is shown that sugar recovery, the overall sugar yield based on carbohydrate in the untreated sludge, for VK2 sludge was higher for fractionated scenario with enzyme dosages higher than 2 FPU/g, Fig. 3.4. At enzyme dosage of 4 FPU/g, the sugar recoveries of non-fractionated and fractionated scenarios were almost the same. This is a consequence of two phenomena: losses of carbohydrates during fractionation and improvements in conversions due to fractionation. Despite the similar sugar recoveries, fractionation has been shown to be useful in order to achieve higher financial returns by lowering the unit inlet flows and production costs (Chen et al., 2014).

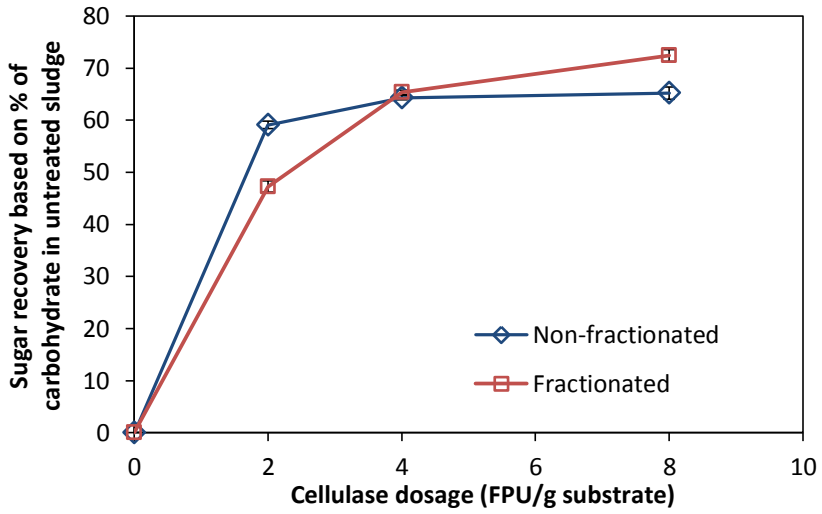

Fig. 3.4 Sugar recovery of non-fractionated and fractionated VK2 sludge. Each data point is the average of two replicates; error bars depict ± 1 standard error of the mean

At the same pH of 5, fractionated sludge generally has a higher sugar conversion than unfractionated sludge (Fig. 3.3 and Table 3.4). This can be explained by the enzyme adsorption on acid insoluble ash components, causing inactive binding during enzymatic hydrolysis, Fig. 3.5. Adsorption results in Fig. 3.5 indicate that the amount of adsorbed enzyme per solid is in the order of bleached hardwood fibers>clay>$CaCO_3$>$CaSO_4$. Increased amounts of enzyme charged produces measurably higher adsorption for the bleached hardwood and clay; the trends for $CaCO_3$ and $CaSO_4$ are much harder to discern at the lower adsorption levels.

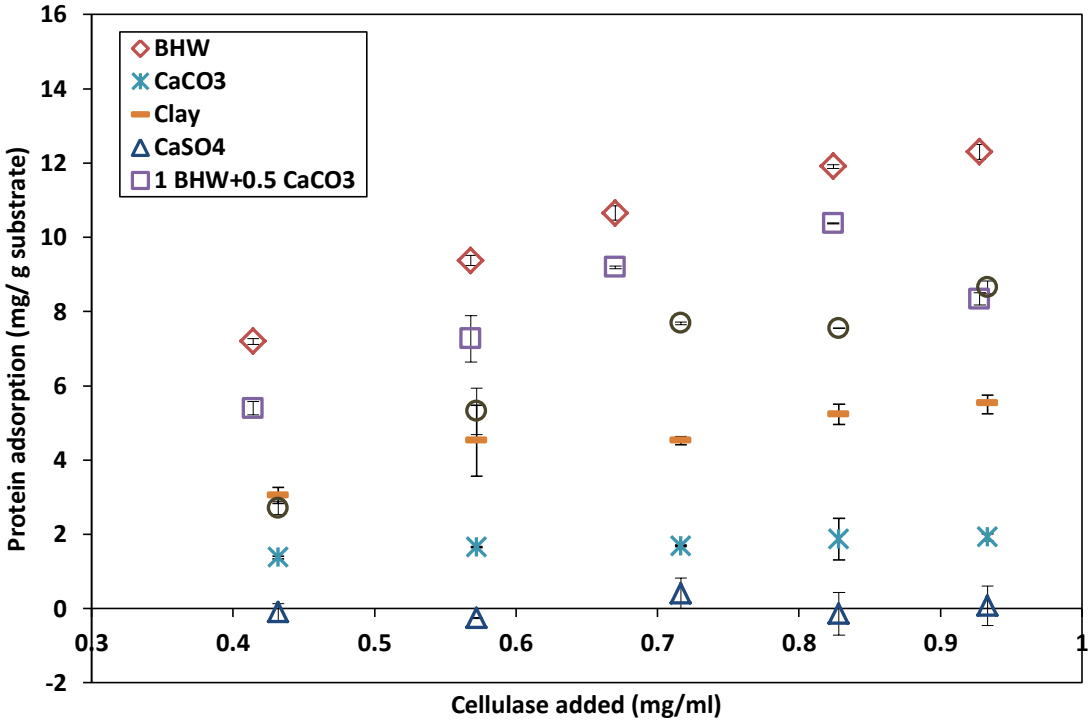

Fig. 3.5 Cellulase adsorbed as a percentage of the total cellulase added on bleached hardwood (BHW), calcium carbonate, and clay and their mixture under different cellulase dosages. Each data point is the average of two replicates; error bars depict ± 1 standard error of the mean

Further, the so-called acid soluble $CaCO_3$ still shows some adsorption of enzyme, probably due to an incomplete solubility at pH of 5 using a buffer. (Fine particles were observed for this condition for $CaCO_3$ in the buffer solution after centrifuging.) Purchased $CaSO_4$, had essentially a zero enzyme adsorption, of interest since it is the conversion product of sulfuric acid and $CaCO_3$. Note that when the $CaSO_4$ was added to the buffer solution that a cloudy suspension was produced, indicating incomplete solubility.

In previous research it was determined that fiber had a higher capacity for enzymes but the ash had a higher affinity for the enzymes (9). To further investigate interactions between

enzymes and competitive adsorption between fibers and ash, combinations of one gram of fiber and 0.5 grams of either clay or $CaCO_3$ were evaluated, Fig 3.5. As expected the total enzyme adsorption lied between the pure fiber and pure ash.

The enzymatic hydrolysates of the fractionated RD1 sludge were fermented with both direct and complex medium addition to produce ethanol. Direct fermentation achieved 92% ethanol conversion based on monosaccharide in hydrolysates with a 4.2 g/l of ethanol product. For fermentation with medium additions, the ethanol conversion was 88% with a 4.3 g/l ethanol product. This indicates that sludge enzymatic hydrolysates have good potential for ethanol production, as would be expected.

Fractionation efficiency improvement

In order to understand the screen opening size effect on the fiber retention and fractionation efficiency, a Britt Jar fractionation method using a small agitated vessel with screen mesh on the bottom with various mesh sizes (100-500 mesh, opening sizes in Table 3.1) was utilized. The Britt Jar utilizes an agitator just above the mesh screen to prevent a mat from decreasing the drainage rate. The overall fractionation results are listed in Table 3.5 for VK2 and RD1. It was shown that the overall yields of total solids were increased for both sludges with increased mesh sizes due to the smaller openings.

Table 3.5 Fractionation efficiencies of VK2 and RD1 sludges with different screen sizes

		100 Mesh		200 Mesh		300 Mesh		400 Mesh		500 Mesh	
VK2	In, g	Ash, % based on retained od wt	Yield, %	Ash, % based on retained od wt	Yield, %	Ash, % based on retained od wt	Yield, %	Ash, % based on retained od wt	Yield, %	Ash, % based on retained od wt	Yield, %
Total	100		38.6		44.2		45.4		50.4		55.4
Ash	50.8	4.3	3.3	5.6	4.9	6.1	5.4	6.4	6.3	14.9	16.2
Fiber	49.2		75.2		84.8		86.8		96		95.9
Ash removal, %		96.7		95.1		94.6		93.7		83.8	
Ash fractionation ratio		0.92		0.89		0.88		0.87		0.71	
RD1											
Total	100		15.8		21.3		23		29.4		30.2
Ash	54.5	2.4	0.7	5	1.9	6.8	2.9	2.4	1.3	29.2	16.2
Fiber	45.5		33.8		44.6		47.1		63.1		47
Ash removal, %		99.3		98.1		97.1		98.7		83.8	
Ash fractionation ratio		0.96		0.91		0.88		0.96		0.46	

Overall yields of ash and organic fiber were based on the original ash and fiber content. For the 100-400 mesh screens, the smaller screen size openings in general decreased the ash removal and increased the yield of fibers retained in the fiber rich stream. The ash fractionation ratios of the screens ranging from 100 to 400 mesh were all above 0.85. In contrast, for the smallest opening screen of 500 mesh the ash fractionation ratio was considerably lower, 0.71 and 0.46 for VK2 and RD1, respectively. Note that the expected filler size of around 0.2-3 microns (Scott, 1996) is much smaller than the openings for even the 500 mesh screen with 25 microns. This suggests that the small openings in the 500 mesh screen developed a fiber mat not completely disrupted by the agitator that acted as a resistance for the filler to pass through the screen. Of the mesh sizes evaluated, the 400 mesh screen is preferred since it had a high ash removal, low fiber loss and high fractionation ratio. These results suggest that in a practical industrial processing situation, that the smallest fractionation opening size may not be the most effective.

The Ash Rich Stream as a Soil amendment

In this study, an ash rich stream from the sludge fractionation process was produced as a byproduct of the fiber rich stream. A preliminary evaluation of the ash rich stream for soil amendment was conducted since it has been shown that paper sludge can be an alternative plant fertilizer (Camberato et al., 2006; Henry, 1991; Jackson et al., 2000). Metal concentrations in the unfractionated paper mill sludges were generally lower than those in municipal waste solids and well within regulatory limits (Camberato et al., 2006), Table 3.6. The major metal compounds of the four unfractionated sludges were within the range for soil amendment compared with other literature values for pulp and papermaking sludges from various sources (Jackson et al., 2000; Mittra et al., 2005; Sims et al., 1995).

Table 3.6 Metal compounds in the unfractionated sludges

Metal compound	VK1	VK2	RD1	RD2
Fe (mg/kg)	1695	1695	1302	1303
Mn (mg/kg)	186	186	34.9	34.6
Zn (mg/kg)	66.2	189	111	112
Cu (mg/kg)	26.5	5.6	26.6	24.9
Cd (mg/kg)	ND	0.62	ND	ND
Cr (mg/kg)	12.7	<5.00	<5.00	<5.00
Ni (mg/kg)	16.6	20.6	<1.00	<1.00
Pb (mg/kg)	10.8	ND	3.25	2.64

Another aspect of the utility of the sludge in soil amendments is the C:N ratio of the sludges. The C:N ratio has often been included as an important factor in determining the rate of mineralization, immobilization and nitrification in models predicting nitrogen turnover and retention in soils (Janssen, 1996). Soils with a high C:N ratio are characterized by rapid

immobilization of N (undesirable) whereas soils with a low C:N ratio produce slower N immobilization with a surplus of available NH_4^+ derived from deamination of organic carbon sources (Bengtsson et al., 2003). In term of applying sludge for soil amendment, the amount and duration of N immobilization during the decomposition of sludge are dependent upon several factors including the amount of sludge, its C:N ratio, soil inorganic N supply and soil type (Camberato et al., 2006).

Paper making sludges have a wide range of C:N ratio reported from 40 to 500 depending on pulp and paper making processes and sludge types (Camberato et al., 2006; Charest et al., 2004; Zibilske, 1987). Primary sludge from clarifiers contains higher carbon (C) and lower nitrogen (N) content compared to secondary sludge from biological treatments. Organic materials with C:N ratios >20–30:1 generally result in some period of N immobilization (Camberato et al., 2006), and primary sludges greatly exceed this ratio. Studies have shown that by applying high C:N ratio sludge on soil, even though for the early times after application there was evidence of undesirable N immobilization, at longer times the pulp and paper sludge improved soil nutrient and crop growth rate (Aitken et al., 1998; Fierro et al., 1999).

In this study, C:N ratios of the ash rich streams from the process were significantly lower relative to the respective unfractionated sludges (VK2 and RD1) and thus would not promote N immobilization as severely as the unfractionated sludge, Table 3.7. Also, the macronutrients were generally preserved in the ash rich streams, Table 3.7.

Table 3.7 pH, C:N ratios and concentrations of macronutrients in unfractionated VK2 and RD1 sludges and their ash rich streams from fractionation

Sludge	VK2	VK2 ash rich stream	RD1	RD1 ash rich stream
pH	8.0	6.5	7.5	6.5
C:N	301.7	201.8	164.9	139.8
% P	0.18	0.28	0.02	0.02
% K	0.08	0.02	0	0
% Ca	17.9	29.1	17.2	21.4
% Mg	0.2	0.13	0.16	0.14
% S	0.13	0.1	0.07	0.05

This supports the claim that the process residue (ash-rich stream) from fractionated sludge to ethanol process is a more suitable material than unfractionated sludge for soil amendment. Also, composting (defined as the microbial degradation of organic solid material that involves aerobic respiration and passes through a thermophilic stage) the ash rich stream or combining it with secondary sludge before utilizing as a soil amendment are also feasible to lower the amount of required supplemental N and improve nitrogen mineralization rates (Charest et al., 2004; Henry, 1991).

Conclusions

A lab scale fractionation process was shown to produce a fiber rich and an ash rich stream. Fractionation of sludge with the removal of inorganic materials (ash) results in increased sugar conversion in enzymatic hydrolysis for the fiber rich stream. This improvement is not simply a result of pH effects since at the same pH fractionated sludge with lower ash content has higher sugar conversion than unfractionated sludge. This finding is in agreement with the observations that enzyme tends to adsorb on insoluble ash

components in sludge. Fractionation also lowered the acid requirement for adjusting the pH for enzymatic hydrolysis by removing ash that consumes acid. Fermentation yields for fractionated sludge hydrolysate were 92% for direct fermentation and 88% with medium complex added fermentation. A fractionation barrier with 400 mesh screen was shown to have the best combination of high fiber yield, high ash removal and high ash fractionation ratio relative to fractionation screens with larger and smaller openings in the range of 100-500 mesh. During the fractionation process, the ash rich stream produced was determined to have improved soil amendment properties (lower C:N ratio with macronutrient preservation) compared to the unfractionated sludge.

Acknowledgments

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Chapter 4 Economic Evaluation of the Conversion of Industrial Paper Sludge to Ethanol

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Abstract

The conversion of industrial paper sludge to ethanol was simulated using engineering process simulation software loaded with laboratory generated conversion data and financially analyzed. In one scenario, sludge is fractionated to remove ash, generating a higher concentration carbohydrate stream for separate hydrolysis and fermentation (SHF). In a second scenario, non-fractionated sludge is processed with only pH adjustment. Four primary sludges from mills producing either virgin or recycled paper were analyzed and the experimental conversion results used to inform the simulations. Financial analysis was conducted assuming ethanol wholesale price of US$ 0.608 per liter. The most profitable case was fractionated virgin sludge (from a virgin paper mill) to ethanol (F-VK1) with a net present value (NPV) of US$ 11.4 million, internal rate of return (IRR) of 28%, payback period of 4.4 years and minimum ethanol revenue (MER) of US$ 0.32 per liter. Risk analysis showed that the F-VK1 case obtained a near 100% probability of business success with both central and bearish (pessimistic) assumptions.

Keywords: Paper sludge, mechanical fractionation, enzymatic hydrolysis, fermentation, process simulation, economic analysis.

Introduction

The level of research for ethanol production from lignocellulosic biomass (second generation biofuels) has become unprecedented with substantial government and private investment, aiming to overcome food-fuel dispute and environmental limitations of first generation biofuels from cereals, grains, sugar crops and oil seeds. The expansion of traditional ethanol production from corn grain results in an increase of corn crop prices. Over 99% of the world's total biofuel production is from first generation processes in 2010, which accounts only for 0.5% of global energy consumption (Schenk et al., 2008). Despite special interest and extensive efforts of government, research institutions, enterprises and universities to improve the competitivity of cellulosic biofuels, several barriers to second generation biofuels still remain. These include: i) lignocellulosic biomass natural recalcitrance that prevents high ethanol yield within tolerable capital expenditure (CAPEX) and ii) high production costs. Also, delivered biomass cost and availability, high pretreatment and chemical cost, intensive CAPEX and overall production costs have been identified as the major obstacles for commercializing cellulosic ethanol with competitive financial returns (Gonzalez et al., 2011a; Hess et al., 2007).

Industrial paper sludge is composed of short pulp fibers from a fraction of the paper making feedstock removed along with clays, fillers and other contaminants (Jeffries & Schartman, 1999; McGovern et al., 1983). As a potential candidate raw material for producing ethanol, paper sludge has certain advantages over other feedstocks such as agricultural residues or wood sources: i) paper sludge is produced at a concentrated site and

permanent production location, making the sourcing of sludge easy at practically no cost; ii) the utilization of sludge for ethanol diverts material going to landfill (avoiding truck hauling costs and landfill investments) and iii) paper sludge is composed of carbohydrate materials in the form of very fine fibers with high specific surface area and often with little lignin present. Since industrial paper sludge has already been subjected to an extensive mechanical and chemical processing (previously imposed during pulping and papermaking processes like cooking, refining and bleaching), polysaccharides in recycled paper sludge are more amenable to enzymatic hydrolysis compared to raw wood or plant material (Keating et al., 2006; Lynd et al., 2001; Wingren et al., 2003). This avoids costly pretreatment to open up the lignocellulosic structure or remove lignin to make it more amenable for enzymatic hydrolysis (Yu et al., 2011).

The process development of sludge to ethanol via a biochemical pathway has been studied over the last few decades (Duff et al., 1994; Fan & Lynd, 2007a; Fan et al., 2003; Lark et al., 1997; Lynd et al., 2001). Previous studies (Kang et al., 2011; Nikolov et al., 2000) show that enzymatic hydrolysis of paper sludge has been inefficient in separate hydrolysis and fermentation (SHF) due to the interference of large amount of ash in the sludges during enzymatic reaction. Our previous work (Chen et al., 2012b) showed that acid soluble ash like $CaCO_3$ not only buffers the pH level (usually 2-3 units higher than the optimum pH) making pH adjustment with acid required for enzymatic hydrolysis, but also adsorbs cellulase with a higher affinity than cellulosic fiber. Acid insoluble ash like clay presents inactive binding with cellulase thereby decreasing enzyme digestibility of fiber in sludge. By fractionating

recycled copy paper it was determined that the enzyme hydrolysis of the fractionated material with 0.6% ash content produced approximately 40% higher sugar than unfractionated material at an enzyme dosage of 4FPU/OD g substrate. In another study (Kang et al., 2011), the fractionation of sludge by using floatation and screening with air and 100 mesh screens, showed a 10% improvement in ethanol yields at an enzyme dosage of 10 FPU/g glucan in a simultaneous saccharification and co-fermentation (SSCF) process. Therefore, in order to achieve higher efficiency in enzymatic hydrolysis (lower enzyme dosage and higher sugar output), a mechanical fractionation approach prior to enzymatic hydrolysis was further investigated in this study. Fractionation of paper sludge lowers total inlet flow into the SHF process thus reducing the reactor sizes needed, and it improves saccharification efficiency by removing a high proportion of ash and impurities that can interfere with enzymatic hydrolysis and fermentation (Chen et al., 2012a). Sugar concentration into the fermentation unit is also higher and thus can result in higher ethanol concentrations in the beer, reducing steam demand for distillation.

Paper mill sludges vary widely because of different feedstocks (e.g., recycled paper, tissue paper, hardwood, softwood) and processes used at different mills. Even among similar mills using similar processes and feedstocks, sludges can vary due to different operating conditions within thermomechanical and chemical unit operations (Mahmood & Elliott, 2006). Therefore, candidate sludges for bioethanol production must be characterized and analyzed before consideration for bioconversion (Lynd et al., 2001). In this work, four sludges from different mills producing either virgin or recycled paper products were

evaluated with both non-fractionated and fractionated scenarios. Process simulations (mass and energy balances) of industrial paper sludge to ethanol processes were constructed based on lab data of fractionation and enzyme conversion efficiency and paper mill data of sludge generation rates. Financial evaluation and sensitivity analysis for both scenarios were presented to identify potential business models for the different sludge conversion processes. This paper fills a gap in the literature regarding a rigorous process economics analysis of using paper sludge for ethanol production as well as the mechanical separation of ash from sludge and its economic impact. This research should therefore provide practical information to researchers, paper industry managers, and investors by considering paper sludge to ethanol as a short term commercial pathway for cellulosic ethanol production.

Materials and Methods

Laboratory experiments

Feedstock

Four types of primary sludge (the primary treatment residue captured in the primary clarifier) were donated from four paper mills in North and South Carolina (USA). Sludges were collected from each paper mill during the summer of 2011 and stored in sealed buckets in a cold room. Candidate sludges were selected from well mixed sludge in the buckets, fluffed and stored in separate sealed plastic zip-lock bags for at least 24 h prior to solid content measurement and subsequent treatments. Composition analyses of all paper sludges were conducted using the NREL standard procedure (Sluiter et al., 2004). Three parallel tests were conducted for all samples. Ash content of sludge was measured using Tappi standard

method, T 211om-85 with a furnace temperature of 575 ℃ (TAPPI, 1993a) and $CaCO_3$ content was measured per Tappi standard method, T 211 om-93 using furnace temperatures of 575 ℃ and 950 ℃ (TAPPI, 1993b). Composition of the sludges is reported in Table 4.1.

Table 4.1 Paper sludge origins, chemical composition and fractionation efficiency

Sludge	Origin		Composition, (wt.%, dry basis)								Fractionation Yield, (wt.%, dry basis)		
I.D.	Furnish[a]	Process[b]	Glucan	Xylan	Mannan	ASL[c]	AIL[d]	Ash	Extractives	$CaCO_3$	Overall yield	Organic[e] yield	Ash removal
VK1	V, HW, SW	K, Paper, Pulp	50.6 (0.1)	9.0 (0.1)	2.61 (0.03)	0.64 (0.002)	10.3 (0.2)	26.2 (0.06)	1.02 (0.2)	24.1 (0.5)	55.8	69.0	81.6
VK2	V,HW, SW	K, Paper	35.6 (0.3)	6.6 (0.1)	1.44 (0.02)	0.54 (0.01)	3.96 (0.09)	50.8 (0.06)	1.05 (0.2)	44.7 (0.1)	35.5	65.5	93.5
RD1	R	D, B, Paper	27.4 (1.1)	6.0 (0.01)	1.40 (0.06)	0.50 (0.07)	6.72 (0.5)	54.5 (0.3)	2.53 (0.1)	49.4 (0.1)	27.7	52.7	93.2
RD2	R	D, B, Paper	25.5 (0.1)	4.7 (0.01)	1.40 (0.1)	0.40 (0.002)	6.74 (0.2)	56.1 (0.2)	2.62 (0.1)	50.6 (0.2)	17.7	38.0	98.2

[a] V = virgin, R = recycled, HW = hardwood, SW = softwood.
[b] D = deinking, K = kraft, B = bleached, Pulp = pulp sludge, Paper = paper sludge.
[b] ASL = acid soluble lignin.
[d] AIL = acid insoluble lignin.
[e] Organic = Total compositions – ash.

Paper sludge fractionation

Fractionation of paper sludge was conducted using a Pulmac Masterscreen (Pul- mac International, Montpelier, VT, USA) with a 0.2 mm hole size opening screen. For individual paper sludge, 30 OD (oven dry) g of sludge sample was first presoaked overnight at 1.5% solid content and dispersed using a disintegrator for 5 min (15,000 revolutions). Program C was used for the Masterscreen fractionation, this program allows for faster cleaning and screening of sludge. A quantity of 500 mL (7.5 OD grams) of sludge suspension was loaded for each run in the Pulmac Masterscreen. Two portions of retained fiber and fine rich streams were obtained after fractionation: (1) primary fiber that would not pass through the 0.2 mm hole screen and were retained on the reject tray and (TAPPI) secondary fiber that passed through the 0.2 mm hole screen but was retained on a 200 mesh (0.074 mm) screen and were

collected. Compositions of original sludges, total recovered material, organic material recovery, and percent ash removal are listed in Table 4.1. Sludges from virgin wood kraft pulping processes were named VK; while sludges from recycled paper deinking process were named RD. Primary and secondary fibers were mixed proportionally according to their yield for enzymatic hydrolysis testing. In general, sludges from virgin paper mills had higher carbohydrate (glucan and xylan) content and less ash content than sludges from recycled paper mills, since the feedstock of virgin paper mills are virgin wood containing high fiber content and no fillers which is in contrast with recycled office type papers with high ash content, Table 4.1. Also, sludge from recycled paper production is expected to have a higher concentration of short fibers and fines relative to sludges from virgin paper production and thus also contribute to lower overall yields.

Enzymatic hydrolysis

Cellic® CTec2 (cellulase complex blended with high level of cellulases, beta-glucosidase, and hemicellulase) and Cellic® HTec2 (endoxylanse with cellulase background) were kindly supplied by Novozymes (Franklinton, NC, USA). Total cellulase activity of 136 Filter Paper Unit (FPU)/mL was confirmed for Cellic® CTec2 using the filter paper assay as described in the International Union of Pure and Applied Chemistry standard method (Ghose, 1987). CTec2 protein content was 160 mg/ml and HTec2 was 40 mg/ml as provided by the enzyme manufacturer. Enzyme dosage was 4 FPU/ OD g sludge and the mixing ratio of CTec2 and HTec2 was 9:1 by volume. A total weight of 2 OD g was immersed in 50 mM sodium citrate buffer (pH of 4.8) and 98% sulfuric acid was added if necessary to adjust the pH to 4.8±0.2.

Enzymatic hydrolysis experiments of paper sludge were carried out in 50mL plastic centrifuge tubes with a solid content of 5% (w/v) at 50 ℃ for 48 h and 180 rpm in an environmental incubator shaker (New Brunswick Scientific, Edison, NJ, USA). Sugar concentrations (glucose, xylose, galactose, arabinose and mannose) for composition analysis and enzymatic hydrolysis results were quantified using a high-performance liquid chromatography (HPLC) system (Agilent 1200, Agilent, Santa Clara, CA, USA). The HPLC system was equipped with a deashing filter (Bio-Rad 125-0118, Bio-Rad, Hercules, CA, USA) and a Shodex SP0810 column (8x300 mm, Showa Denko, Tokyo, Japan). The mobile phase was Milli-Q water at a flow rate of 0.5 mL/min and temperature of 80 ℃.

Process simulation

A complete process model for the industrial paper sludge to ethanol biorefinery was built using WinGEMS v5.3. This simulation software was developed for the pulp and paper industry and is comprised of modular blocks for unit operations, process streams connecting the unit operations, an executive program administrating the solution of the streams, and a database of physicochemical and thermodynamic properties of components. When all the streams and blocks are built, it runs to solve the mass and energy balance of the whole model by iterative calculations. The iterations stop when the convergence criteria are reached.

A basic high-level process flow sheet for the sludge to ethanol process is illustrated in Fig. 4.1. This is a simplified flowsheet, which does not show all of the modular blocks used in the WinGEMS simulation.

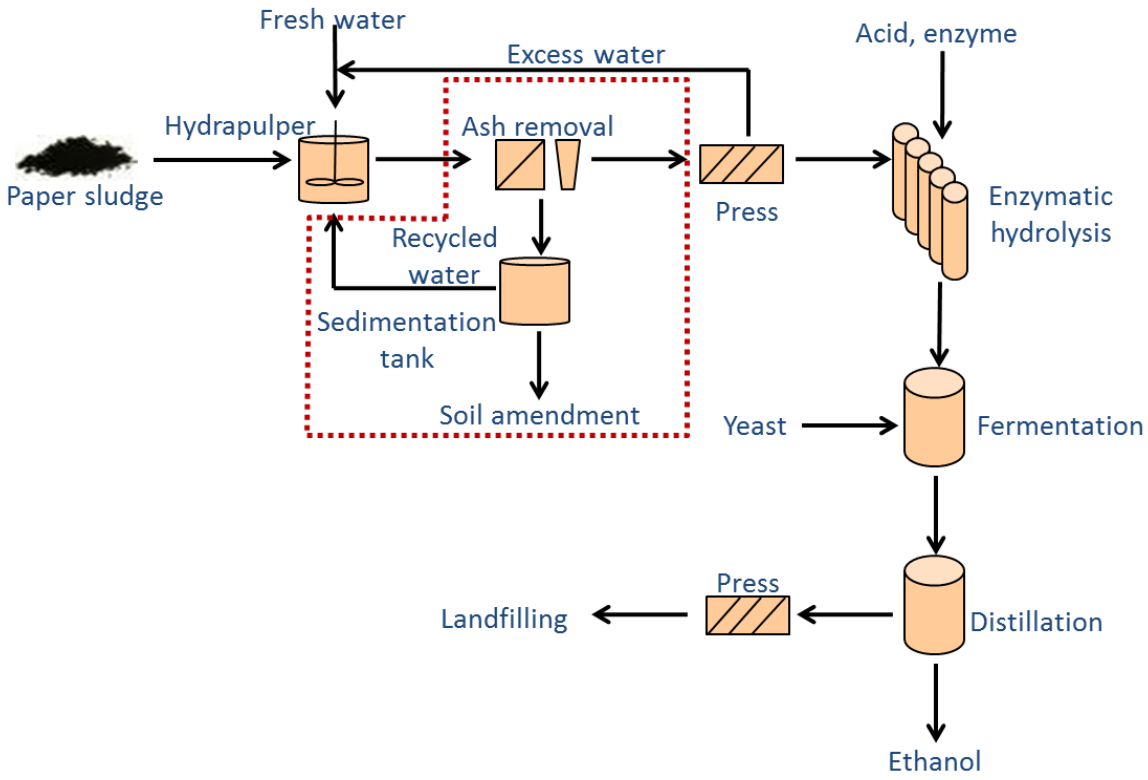

Fig. 4.1 Sludge to ethanol process flow sheet (red dotted area is the fractionation process and the difference between fractionation and non-fractionated scenarios)

The fractionated sludge to ethanol process consisted of seven major unit operations including paper sludge pulping using a hydrapulper, fractionation by side hill screens, sedimentation of the solids in the filtrate from the fractionation, pressing by a screw press, enzymatic hydrolysis, fermentation, distillation and pressing of residue from the beer column bottom by another screw press. Enzymatic hydrolysis was simulated at 15% consistency and 48 h retention time, since lab data and previous studies have shown that conversion difference was marginal when increasing the consistency from 5% to 15% (Xue et al., 2012). The non-fractionated scenario had all these unit processes except fractionation (as shown in

the dotted frame). Both fractionated and non-fractionated sludge were pH adjusted to 4.8 with sulfuric acid prior to enzymatic hydrolysis.

Financial modeling

Design context

Four types of primary paper sludges were obtained from the underflow of a primary clarifier with average solid contents of 30% as material has been dewatered by presses before being landfilled or incinerated. In this study, landfilling was selected for all cases regarding the sludge disposal option for the mill as well as for the process residue from ethanol plant. Therefore the base case for paper mill sludge disposal cost becomes a savings for the ethanol production business units. According to FisherSolve™, VK1, VK2 and RD1 mills produce ~75, 25 and 20 BDT (bone dry short tons) of sludge per day, respectively (RD2 mill is currently shut down). Since VK1 sludge is the most promising sludge source for ethanol production, the sludge inflow rate for all cases was set to be 3 BDT or 2.7 dry metric tons per hour for comparison purposes, equivalent to 22,680 dry tons per year.

The ethanol plant is expected to be sited within the paper mill, thus no land cost was considered. However, a land preparation cost associated with setting up the area for the ethanol plant was taken into consideration. Fresh paper mill water was sourced to the ethanol plant as cooling water and process water. Available resources like electricity and steam were purchased directly from the paper mill. Since unfermentable substrate and residual ash from the beer column bottoms were transferred back into the mill wastewater treatment system

after the primary clarifier, the ethanol plant does not need its own raw water treatment system or waste water treatment system.

General assumptions

Major assumptions applied in the financial analysis are listed in Table 4.2. Operational parameters like sludge inflow rate, fermentation efficiency of C5 and C6 (5-carbon and 6-carbon) sugars (80% and 95%, respectively) were assumed for the WinGEMS simulation. The startup year was assumed to be 2013 with 15 years of project life after the startup year. Construction and spending was assumed to begin in 2012. Total annual operating hours were assumed to be 96% availability (8,400 hours per year). A depreciation schedule of 10 years straight line was used in the analysis with a 12% discount rate and 35% tax rate. For a bio-refinery with low risk in terms of technology, the discount rate is typically ~6% (Treasure et al., 2012), however, due to the fact that this technology has not been demonstrated on a commercial scale the discount rate of 12% has been used which is consistent with other studies (Aden et al., 2002; Frederick Jr et al., 2008; Gonzalez et al., 2012; Gonzalez et al., 2011a; Gonzalez et al., 2011b).

Table 4.2 Modeled chemical and utility cost, conversion efficiency, and major assumptions used in the economic analysis

Description	Value	Description	Value
Startup Year	2013	Sludge Moisture Content, % of Green Ton	70%
Terminal Year	2027	Biomass Cost, $ per Ton	0.0
Feedstock supply, dry tons/year	22680	Sulfuric Acid Cost, $ per Ton	72
% of Spending in Year -1	80%	Steam Cost, $ per 1000 pounds	5
% of Spending in Year 0	20%	Power, $ per MWH	50
% of Nominal Capacity, Project Year 1	80%	Enzyme Cost, $ per kg	1.5
% of Nominal Capacity, Project Year 2	90%	Yeast Cost, $ per Liter Ethanol	0.018
Excess Material Use in Project Year 1	30%	Hourly and Administrative Staff (Non-Maintenance)	2
Working Capital, % of Direct Cost + Product Revenue	10%	Salaried Staff	2
Annual Increase of Replacement Asset Value (RAV)	3%	Non-Maintenance, $ per Person per Year	45,000
Years Depreciation Schedule, Straight Line	10	Staff, $ per Person per Year	75,000
Tax Rate, with Tax Loss Carryforward	35%	Maintenance Expense, % of RAV [b]	1.0%
Discount Rate	12%	Capital Reinvestment, % of RAV	0.5%
Terminal Value, Year 15 EBITDA Multiple [a]	7	Other Fixed Costs, % of Sales	1.0%
Hours per Year	8400	Sales and Other Overhead, % of Sales	1.0%
Ethanol Revenue, $ per Liter	0.608	Enzymatic Hydrolysis Annual Improvement	2.0%
Subsidy added to price, $ per Liter Ethanol	0.0	Hydrolysis residence time	48h
Subsidy, Tax Credit	0.0	Fermentation efficiency C6	95%
Landfill Cost Avoidance, $ per Wet Ton Sludge	0.94	Fermentation efficiency C5	80%

Note: All prices are expressed as US dollars unless specified.
[a] EBITDA = Earnings Before Interest, Taxes, Depreciation, and Amortization;
[b] RAV = Replacement Asset Value

In years where no profit was made, taxes were calculated as 35% of the negative profit in that year that offset part of the taxes of the paper mill. A terminal value in year 15 of seven times of EBITDA (Earnings Before Interest, Taxes, Depreciation, and Amortization) in the same year was assumed. Sludge was assumed to be no cost, and a cost benefit was assumed for reduction of landfill, as explained above. Sludge from the mill was assumed loaded into the process with 30% solids content. Ethanol yield (liters per dry ton sludge) was assumed to increase at 2% annually as learning curve and enzyme product development progress (if the annual increasing rate is increased by 1%, IRR will be increased by 1%). Sludge landfill cost per wet ton was US$ 0.94, estimated from mill data (sludge density 65 lb/ft^3 and landfill cost US$ 75 per 100 yard^3).

Ethanol price, enzyme costs and dosage

Ethanol selling price was assumed to be US$ 2.30 per gallon (US$ 0.608 per liter) according to the average FOB, Omaha, NE ethanol selling price of 2011 and the 1st quarter of 2012 (NEO, 2012) and was assumed to increase at a rate of 2% per year. The selling price increase is in line with other researchers assumptions (Gonzalez et al., 2012; Gonzalez et al., 2011a). No ethanol subsidy or tax credit was used in this analysis. Enzyme dosages for all evaluation cases were 4 FPU/ OD g of sludge. The enzyme cost was estimated at US$ 1.5 per kg of enzyme solution as provided by the manufacturer, and it was assumed to have an annual decrease in cost of 1%. A detailed enzyme cost calculation is shown in Table 4.3. It was based on sugar conversion of VK1 case.

Table 4.3 Enzyme cost calculation demonstration (case of VK1)

	A	B	C	D	E	F	G	H	I
1	Description	% of dry sludge	EH efficiency	To EH (Kg/Kg)	Stoich	Kg Sugar	Fermentation Efficiency	Stoich	Kg EtOH
2	Glucan	50.6%	57.4%	0.506	111%	0.323	95.0%	51%	0.157
3	Xylan	9.0%	79.4%	0.090	114%	0.081	80.0%	51%	0.033
4	FPU / ml enzyme product	136							
5	$/kg Enzyme Product	1.5							
6	Dosage: FPU/od g sludge	4							
7	gal ethanol/kg ethanol	0.327							
8	g enzyme / ml enzyme product	1							
9	enzyme cost ($)/gal ethanol	0.710							

A9=((B6*1000/B4)*B8/1000*B5)/((I2+I3)*B7)

Note: grey cell is factor; white cell is calculated value and red cell is the target value.

Table 4.4 Sugar conversions, ethanol yields, enzyme costs for fractionated and original paper sludge to ethanol at 4 FPU/OD g sludge and their percent with respect to assumed ethanol selling price

Scenarios	Sugar conversion % of carb in sludge	Ethanol yield liters/dry ton sludge	Enzyme cost US$/gallon ethanol	Enzyme cost US$/liter ethanol	% of ethanol price at US$ 0.608/liter
VK1	59.5	243.2	0.710	0.183	30.1%
VK2	64.3	183.4	0.949	0.245	40.3%
RD1	67.1	153.9	1.134	0.293	48.1%
RD2	94.7	202.9	0.879	0.227	37.3%
F-VK1	81.9	244.6	0.390	0.101	16.6%
F-VK2	91.5	188.9	0.208	0.054	8.8%
F-RD1	78.8	78.0	0.386	0.100	16.4%
F-RD2	89.3	93.5	0.304	0.078	12.9%

Note: F- is the cases with fractionation process.

The sugar conversions, ethanol yield and enzyme costs for the four sludges in the two scenarios are shown in Table 4.4. Generally, the fractionated scenario obtained higher sugar conversions. Ethanol yield was based on the liters of ethanol production per dry ton of untreated starting material. Sludges from virgin paper mills generally had higher overall ethanol yield than sludges from recycled paper mills due to their higher carbohydrate recovery in the fractionation process. For sludges from virgin paper mills, fractionation prior to enzymatic hydrolysis reduced enzyme dosage to achieve the same ethanol yield compared to the process without fractionation, reflecting in the lower enzyme cost per gallon/liter of ethanol in the fractionated sludge to ethanol scenario.

CAPEX

Two scenarios are evaluated for capital investment: fractionated and non-fractionated sludge to ethanol. Capital expenditure was based on data sources from the NREL dilute acid report (Humbird et al., 2011), quotes from vendors and consultation with financial experts. All CAPEX and costs were indexed to 2013 US dollars. Detailed CAPEX information

(taking the F-VK1 case as an example) such as equipment cost, source, installation factor and others are depicted in Table 4.5.

Table 4.5 Capital expenditure for F-VK1 case

Description	Source	Base year	Scaled cost, US$	Installation factor	Ratio index to year 2013	Number required	Total installed cost in 2013, US$
Land preparation	Mill study	2010	432,000	1.0	1.09	1	469,087
Sulfuric Acid Pump	NREL	2010	848	2.3	1.09	1	2,117
Sulfuric Acid Storage Tank	NREL	2010	923	3.0	1.09	1	3,006
Pulper	Vendor	2012	179,000	2.8	1.02	1	510,268
Sidehill Screen	Vendor	2012	11,887	3.0	1.02	3	108,916
Screw Press	Vendor	2012	65,644	1.5	1.02	2	200,495
Screw Press	Vendor	2012	65,934	1.5	1.02	1	100,690
Enzymatic hydrolysis	Mill study	2005	418,748	1.5	1.27	1	799,013
Fermentation	NREL	1998	452,848	2.1	1.54	1	1,468,489
Beer column	NREL	1998	40,111	2.1	1.54	1	130,071
Rectification column	NREL	1998	114,852	2.1	1.54	1	372,439
Dehydration	NREL	1998	305,837	1.0	1.54	1	472,269
Product storage and shipment	NREL	1998	58,254	1.4	1.54	1	125,938
						Sum (TIEC)	4,762,797
						10%Engineering	476,280
						1%Legal Expenses	47,628
						17%Contractor Fee	809,676
						10%Contingency	476,280
						Total capital cost	6,572,660

Sensitivity and risk analysis

Sensitivity and risk analysis was performed using the probability modules of the @RISK software. The goal of the sensitivity analysis in this paper is to provide an estimated value of the probability of success or failure of the entire project. The sources of variation are ethanol wholesale price, ethanol yield (liters of ethanol per dry ton of feedstock), CAPEX and enzyme cost. Ethanol selling price distribution was adjusted following a Weibull distribution, using the distribution fit modules of @RISK software (500 iterations for all cases). Probability distributions of enzyme cost, CAPEX and ethanol yield were fitted using a uniform distribution. Two scenarios were established, central scenario and bearish scenario.

Central scenario included variation of +/- 25% of CAPEX, +/- 25% of ethanol yield and +/- 25% of enzyme cost; while in the bearish scenario the following variations were assumed: CAPEX -15%/+25%, ethanol yield -25%/+15% and enzyme cost -15%/+25%.

Results and discussion

Simulation output

Simulation output of VK1 sludge for both scenarios (non-fractionated and fractionated) from WinGEMS is illustrated in Table 4.6. Since in the fractionated scenario the ethanol concentration before distillation was higher and the inlet flow of SHF was lower relative to the non-fractionated scenario, the fractionated sludge to ethanol process required a total steam requirement of 1.60 mt/hr for heating up the streams for enzymatic hydrolysis and distillation. This was much lower than the total steam requirement (2.42 mt/hr) for the non-fractionated scenario. Enzyme consumption was smaller in the fractionated scenario since a majority of ash was removed before enzymatic hydrolysis. The fractionated scenario also required less demand of sulfuric acid to adjust the pH of the system. RD1 sludge shows the same trend. Generally, sugar and ethanol concentrations from recycled sludge were lower compared to virgin sludge in both scenarios. This was reflected in lower steam consumption and equipment inlet flows. Since recycled sludge contains higher ash content, it required around 50% higher sulfuric acid for neutralization.

Table 4.6 Simulation output of sludge to ethanol process for both scenarios using VK1 and RD1 sludge as feedstock

Sludges	VK1		RD1	
Parameters	N [a]	F [b]	N	F
Steam Consumption, mt/hr	2.42	1.60	1.67	0.66
Enzyme Consumption, mt/hr	0.09	0.05	0.09	0.03
Sulfuric Acid Consumption, mt/hr	0.70	0.13	1.32	0.24
Distillation Residue, mt/hr at 30% Solid Content	2.67	0.92	2.10	0.54
Enzymatic Hydrolysis Inlet Flow, mt/yr	102627	66797	73268	26274
Fermentation Inlet Flow, kg/hr	13231	8964	9263	3448
Beer Column Inlet Flow, kg/hr	12736	8469	9052	3291
Rectification Column Inlet Flow, kg/hr	12099	8046	8600	3126
Dehydration Inlet Flow, kg/hr	1230	1229	770	390
Ethanol (95%) Production, kg/hr	494.5	494.0	309.6	157.0
Inlet Flow to First Screw Press, mt/hr	88.2	13.5	88.2	6.7
Inlet Flow to Second Screw Press, mt/hr	11.5	7.2	8.3	2.9
Waste Water, kg/hr	1510	830	2280	590
Sugar Concentration Before Fermentation, % (w/w)	8.5	12.6	7.6	10.3
Ethanol Concentration Before Distillation, % (v/v)	5.4	8.1	4.8	6.6

[a] N: non-fractionated sludge to ethanol process;
[b] F: fractionated sludge to ethanol process.

In this simulation, the process residue was removed from the beer column bottom and thickened to 30% solid content by a screw press. This design can have zero loss of sugar compared to building a filtration unit before fermentation to remove enzymatic hydrolysis residue. It was shown that the fractionated scenario produced less distillation residue compared to the non-fractionated scenario, therefore had less waste disposal penalty. Process flows (inlet flows of enzymatic hydrolysis, fermentation, screw press etc.) were the basis for CAPEX estimation of each piece of equipment as well as waste water treatment. Although waste water treatment in this case was covered by the paper mill, it would be ~1% of the total CAPEX for this case if a greenfield mill is designed. Since the process flows for the

fractionated scenario were lower than those of the non-fractionated scenario, total CAPEX was decreased for the fractionated scenario.

Ethanol yield and profitability

Ethanol production (million liters per year), net present value (NPV), payback period (years) and internal rate of return (IRR) for both scenarios with 22,680 dry tons per year of sludge are illustrated in Fig. 4.2. Ethanol production ranged from 1.8 to 5.6 million liters per year. Sludges from recycled mills produced lower annual ethanol output in the fractionation scenario due to high fiber loss during the fractionation stage. Payback period is the number of years required to recover the initial financial outflows (Cooper & Kleinschmidt, 1987). The shortest payback period occurred for the fractionated VK1 sludge (4.4 years) with the highest NPV and IRR (US$ 11.4 million and 28%, respectively). In general, all fractionated sludge to ethanol processes had positive NPV, high IRR and payback period ranging from 4.4 to 8.8 years. Except for the VK1 sludge, all non-fractionated processes had negative NPV, and the payback periods were all over 10 years. Although VK1 sludge achieved the same ethanol yields for both non-fractionated and fractionated scenarios, the fractionated scenario obtained higher NPV and IRR since its CAPEX and production costs were lower compared to the non-fractionated scenario. This is mainly due to smaller downstream equipment sizes and lower demand for enzyme and chemicals. For the most profitable case, F-VK1, a modified internal rate of return (MIRR) was estimated (Kierulff, 2008), assuming an 8% reinvestment rate for cash flows. Compared with its IRR of 28%, the MIRR was estimated to be 19% based on the same discount rate (12%). When the selling price assumption is changed from 2% annual

increase (as used in Fig. 2) to 0% (data not shown), the IRR decreases about 2-3% for all cases but the relative order of the profitability of the different cases remains the same.

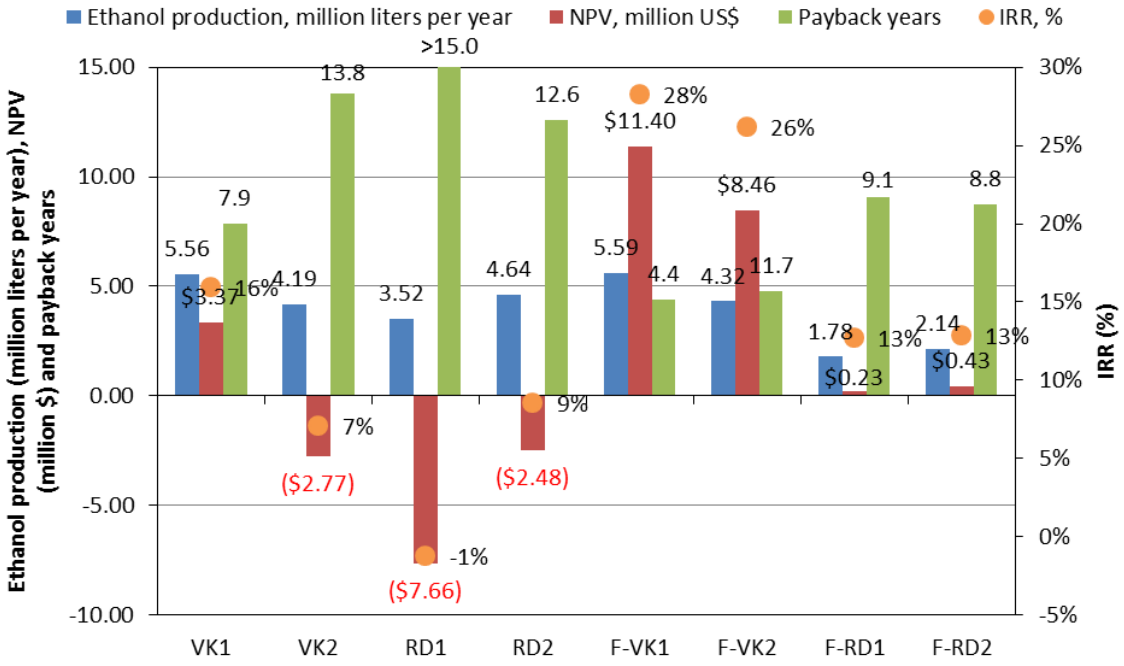

Fig. 4.2 Ethanol production (million liters per year), NPV (million US$), payback period (years) and IRR (%) for each case

Production costs

Production costs include feedstock (in this case zero), operating costs (process chemicals, enzyme etc.), and fixed operating costs (employee salaries, overhead, maintenance etc.). Based on the results from Fig. 4.2, the most profitable mill cases for fractionated and non-fractionated scenarios were VK1 sludge to ethanol. In order to identify the cost drivers of the fractionated and non-fractionated scenarios, production cash costs of VK1 sludge using both processes in year 2015 were analyzed, Fig. 4.3(a) and (b). It is shown that the non-fractionated process had seven times higher chemical cost (mainly sulfuric acid) compared to

the fractionation process, and 1.8 times higher enzyme costs and 1.5 times higher energy costs. In terms of ethanol production cost share, the fractionated sludge to ethanol process had enzyme costs of 46%, energy costs of 16% and chemical costs of 6% of the total production cash cost (production cost minus non cash costs such as depreciation). The non-fractionated process had enzyme costs of 48%, energy costs of 13% and chemical costs of 19% of the total production cash cost. Cash cost for the fractionated process was around US$ 0.22 per liter of ethanol ($0.82 per gallon), whereas total production cost (depreciation of US$ 0.11 per liter of ethanol included) was estimated at US$ 0.33 per liter of ethanol ($1.25 per gallon). Cash cost for the non-fractionated process was around US$ 0.39 per liter of ethanol ($1.46 per gallon), whereas total production cost (depreciation of US$ 0.14 per liter of ethanol included) was estimated at US$ 0.53 per liter of ethanol ($2.00 per gallon).

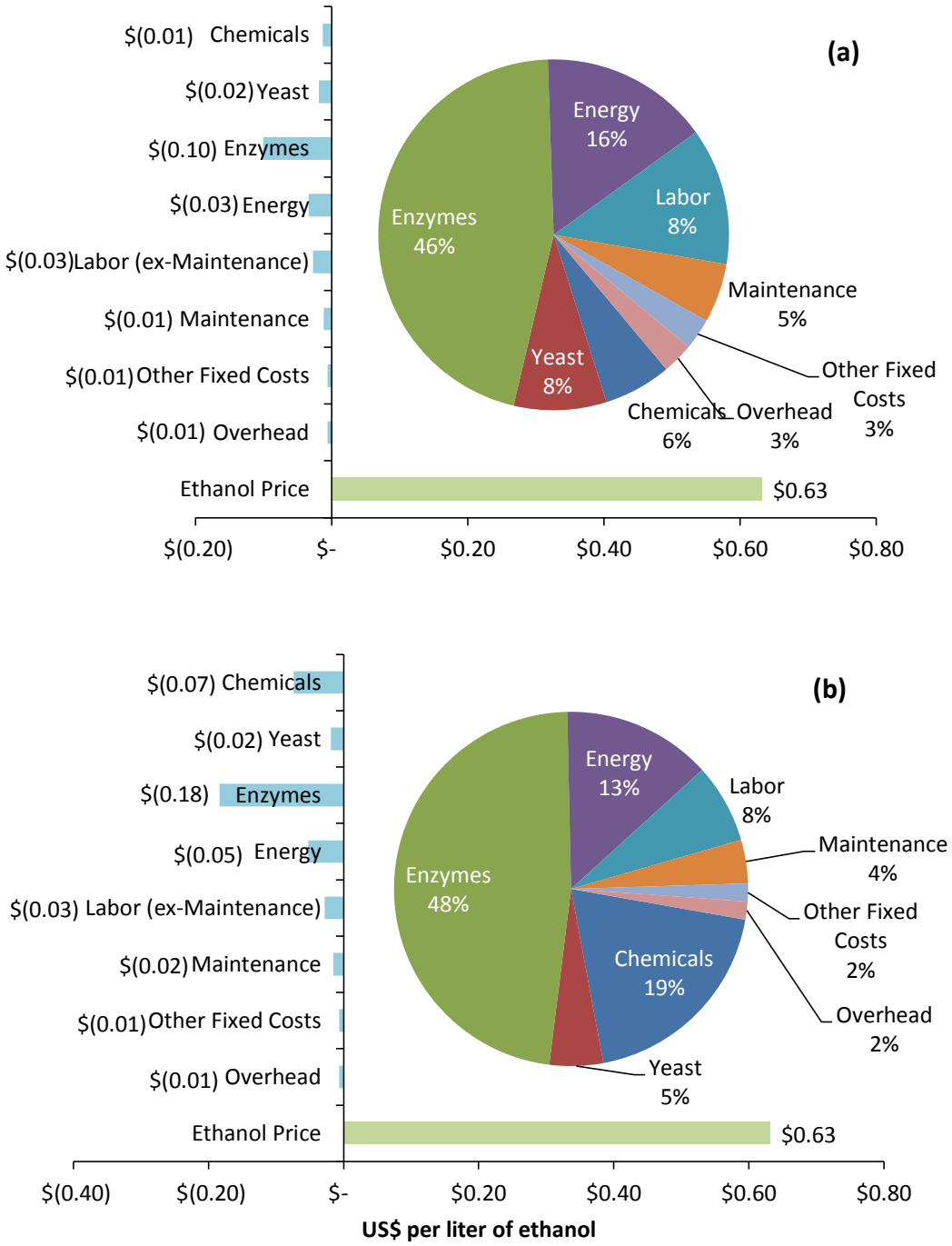

Fig. 4.3 Ethanol production cash costs (US$ per liter ethanol) and cost share (%) for both scenarios using VK1 sludge as feedstock in 2015. (a) fractionated sludge to ethanol; (b) non-fractionated sludge to ethanol

Minimum ethanol revenue (MER) and CAPEX

Total capital expenditure (CAPEX) and CAPEX per liter of ethanol for both scenarios are illustrated in Fig. 4.4 along with the minimum ethanol revenue (MER). Generally, non-fractionated processes required higher CAPEX compared to fractionated sludge to ethanol processes. Since processes with fractionation of recycled sludges had low carbohydrate yields during ash removal, F-RD1 and F-RD2 presented the highest CAPEX per liter of ethanol production (US$ 2.11 and 2.89 respectively). Fractionation of virgin sludge had the lowest CAPEX per liter of ethanol (US$ 1.17 per liter for F-VK1). For comparison, a previous sludge to ethanol study showed a total capital cost of US$1.24 million for a scenario that xylan conversion and mineral recovery were not considered. For another scenario with these considered, a total capital cost of US$2.61 million was stated. In that study, the sludge inflow rate was 15 dry ton per day (Fan & Lynd, 2007b). In terms of woody biomass, a study on the conversion of Eucalyptus (hardwood) to ethanol in a co-current dilute acid process achieved ethanol yield of 347.6L/ dry ton of biomass and a CAPEX of ~$1.03 per liter of ethanol (Gonzalez et al., 2011b). In another study, applying green liquor pretreatment in a greenfield ethanol plant, natural hardwood and Eucalyptus had a CAPEX of $2.4 per liter of ethanol, and pine had a CAPEX of $2.5 per liter of ethanol (Gonzalez et al., 2011a).

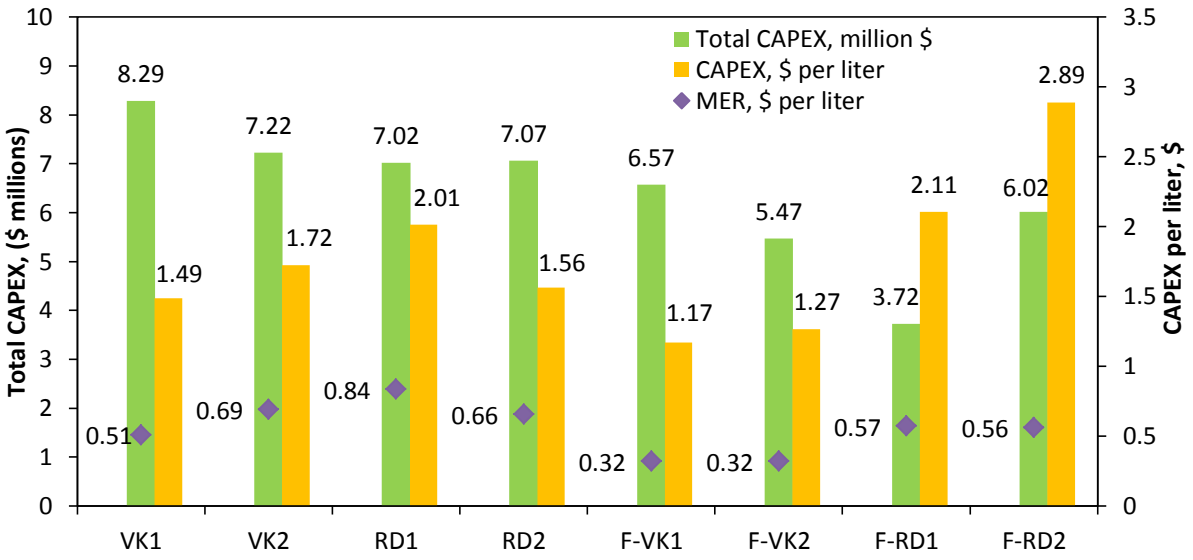

Fig. 4.4 CAPEX (million US$), CAPEX per liter (US$) and MER (US$ per liter of ethanol) for each case

MER is defined as the price (US$ per liter of ethanol) required to achieve a zero NPV at a defined discount rate, in this case 12%. As expected from the discussion of CAPEX and operating costs, fractionated sludge to ethanol scenarios obtained lower MER values. F-VK1 had the lowest MER at US$ 0.32 per liter of ethanol ($1.18 per gallon). Since the ethanol selling price assumed in this study was US$ 0.608 per liter, all the fractionated sludges to ethanol plus VK1 presented MERs lower than the ethanol selling price. For comparison, MERs ranging from ~US$0.74–1.05 per liter of ethanol were calculated in the study of co-production of bioethanol and electricity (value prior to combustion) from mixed southern hardwood and southern yellow pine (Treasure et al., 2012).

In addition, it is of interest to note that although F-VK1 and VK1 had almost the same ethanol yield, VK1 required $8.3 million total CAPEX which is 26% higher than the $6.5

million CAPEX of F-VK1, resulting in lower profitability for VK1. This was mainly due to the fact that fractionation of sludge brought lower process flows into the SHF and distillation units, thus decreasing the required size of the equipment compared to non-fractionated process.

Sensitivity analysis

Sensitivity to gasoline price

The feasibility of this work is highly influenced by ethanol wholesale price. Excluding subsidies, and the "invisible hand" of the market, ethanol price is assumed to be 65% of the value of gasoline price based on energy density (Demain, 2009). The financial sensitivity of IRRs to the ethanol price is illustrated in Fig. 4.5. The corresponding gasoline wholesale price and the historical crude oil price are also indicated. The IRRs of fractionated processes have higher increase rates with the increase of ethanol/gasoline price relative to non-fractionated sludge processes. F-VK1 and F-VK2 would have had over 20% IRR in the year 2011 with the crude oil price of US$ 95 per barrel. Except for the VK1 sludge, other non-fractionated sludge processes had no economic feasibility in the near future unless the gasoline price reaches US$ 1.5 per liter. Although VK2 sludge is from a paper mill processing wood to virgin paper, its ethanol yield was lower than VK1 sludge, presumably due to its shorter fibers that resulted in lower fractionation efficiency.

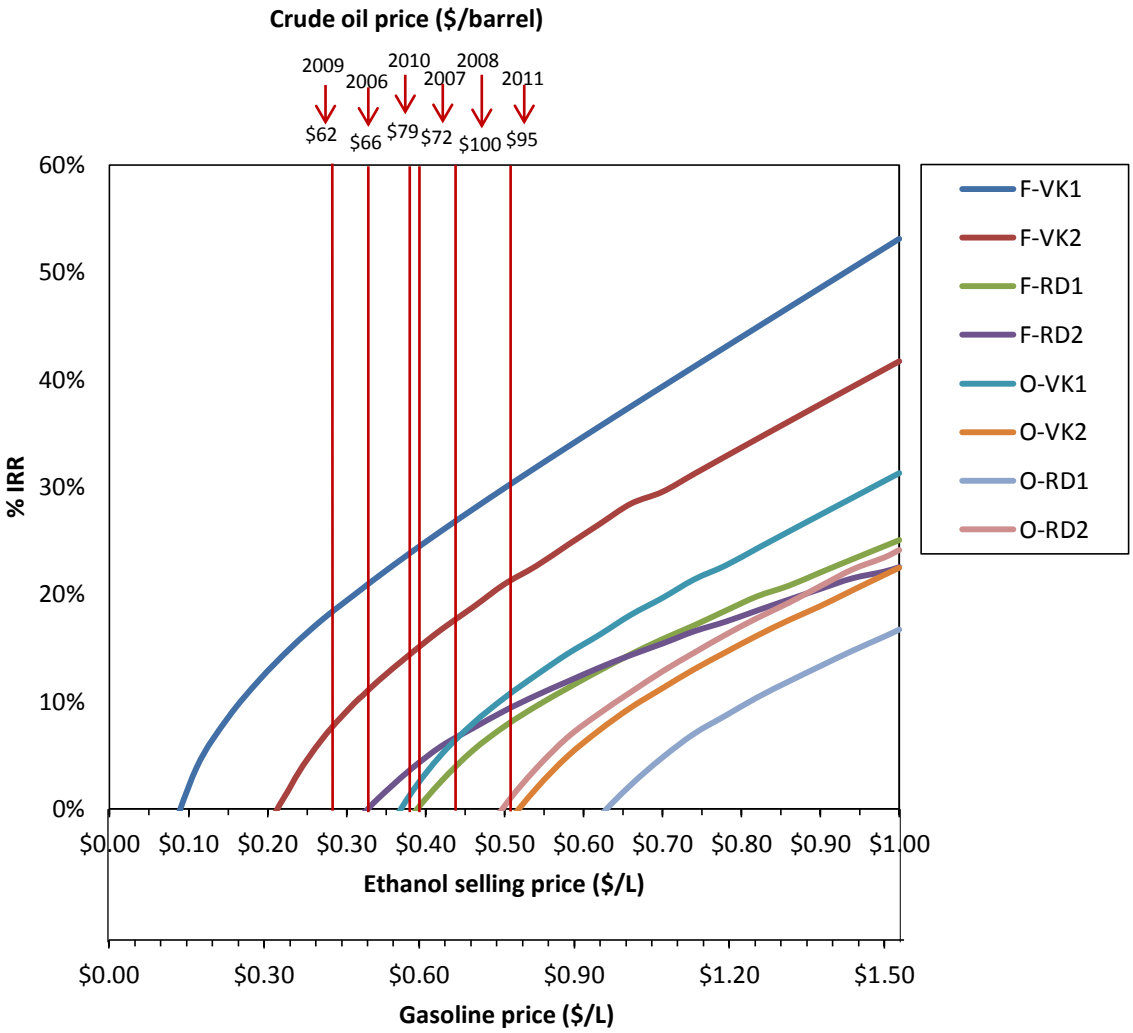

Fig. 4.5 Internal rate of return as a function of ethanol selling price and gasoline price for all cases. Gasoline price reference corresponds to annual averages for Omaha, NE ethanol price FOB (www.neo.ne.gov). Crude oil price reference corresponds to annual averages for Cushing, OK West Texas Intermediate Spot Price FOB (www.eia.doe.gov)

Sensitivity to cost drivers

In order to understand the effect of the magnitudes of cost drivers on minimum ethanol revenue (MER), a sensitivity analysis was performed varying sludge input (process equipment sizing to accommodate a given nominal sludge inflow rate), ethanol yield, ethanol

price, enzyme costs and chemical costs, Fig. 4.6. These variables were manipulated by +/−25% for VK1 sludge for both fractionated and non-fractionated scenarios. The fractionated VK1 sludge to ethanol process was influenced most significantly by changes in sludge input rate (biomass input), whereas the non-fractionated scenario was most sensitive to ethanol selling price. All the other fractionation cases were most sensitive to sludge inflow rate and all the other non-fractionated cases were most sensitive to ethanol selling price (analyses not shown). Ethanol price is controlled by various market factors that cannot easily be affected by a single production facility. In contrast, the designed process sludge inflow rate can be manipulated (meaning sludge loading can be increased by sizing up equipment of the ethanol plant and sourcing additional sludges).

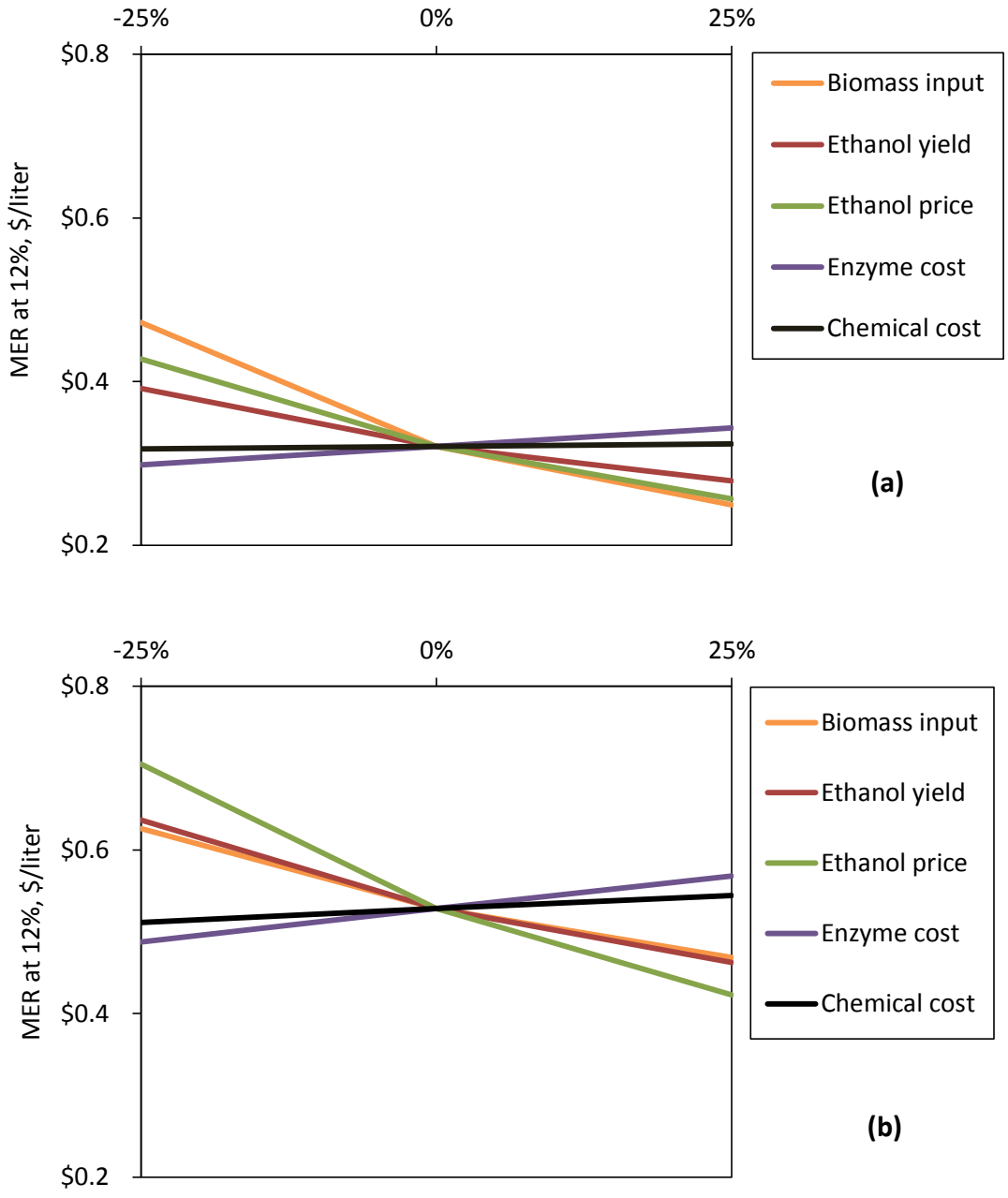

Fig. 4.6 Sensitivity analysis +/−25% in biomass input (sludge inflow rate), investment (CAPEX), ethanol yield, ethanol price, enzyme costs and chemical costs using VK1 sludge as feedstock. (a) fractionated sludge to ethanol; (b) non-fractionated sludge to ethanol

Risk analysis

A risk analysis for the F-VK1 case was conducted. The probability distribution of ethanol wholesale price was simulated using a Weibull distribution with an average value of US$2.28 per gallon (period between Jan. 2005-Dec. 2011) with ca. 98% of the values ranging from $1.6 to $3.2 per gallon, Fig. 4.7(a). It is shown that in the central scenario, the NPV distribution for F-VK1 case had a probability of 99.8% for an IRR value higher than 12%, Fig. 4.7(b). Even in the bearish scenario, the NPV distribution was still almost 100% probable to achieve an IRR higher than 12%, even though the average NPV was decreased by US$1.5 million compared to central scenario, Fig. 4.7(c). This analysis indicates that the proposed process and business model have very high probability of success under current market conditions.

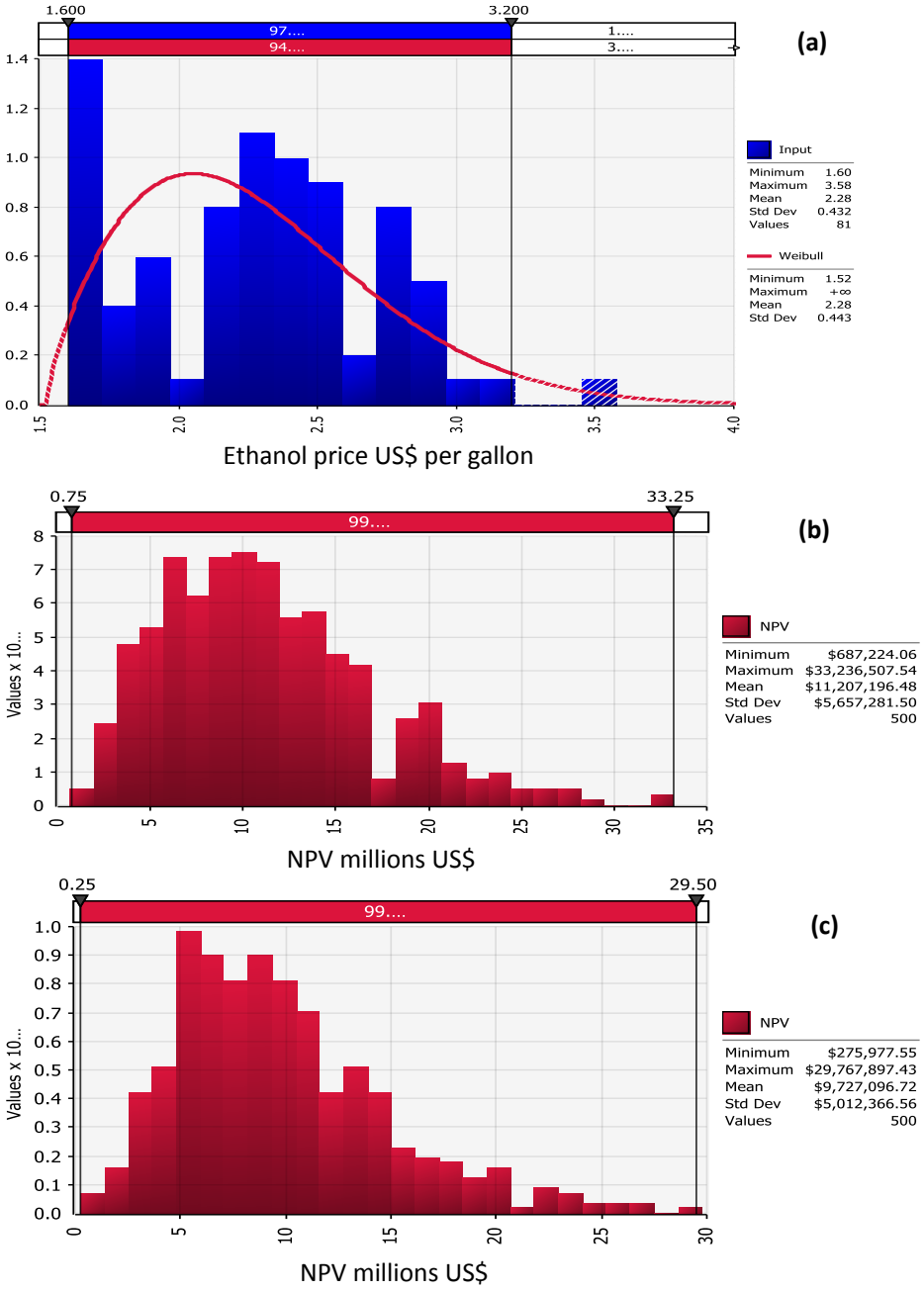

Fig. 4.7 (a) Distribution probability of ethanol prices based on data from Omaha Nebraska wholesale price in the period Jan. 2005-Dec. 2011; (b) distribution of NPV (discount rate 12%) central scenario, F-VK1 case; (c) Distribution of NPV (discount rate 12%), bearish scenario, F-VK1 case

Conclusions

The fractionated sludge to ethanol scenario was determined to be more profitable (even with the same ethanol yield) compared to the non-fractionated scenario due to lower CAPEX and production costs. Sludges acquired from virgin paper mills were determined to have higher fractionation efficiency than sludges acquired from recycled paper mills. The lowest MER was US$ 0.32 per liter (ethanol revenue US$ 0.608 per liter) for fractionated virgin sludges. The MER of the fractionated scenario was most sensitive to sludge inflow rate, while the MER of the non-fractionated scenario was most sensitive to ethanol wholesale price. Risk analysis indicates a very high probability of success (99.8%) to commercialize F-VK1 under current market conditions with both central and bearish scenarios.

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Chapter 5 Enzymatic Hydrolysis of Pretreated Newspaper for Ethanol Production

Abstract

Paper materials with high levels of lignin have been found to be difficult to convert to sugar via enzyme hydrolysis. In order to address this issue, several pretreatments of newspaper substrate prior to enzymatic hydrolysis were evaluated as methods to improve sugar yields. A simple hot water treatment, autohydrolysis, was identified to adversely affect sugar conversions. This was presumably due to the pore collapse under high temperature pretreatment of the newspaper fibers. Flexo ink used in newspaper printing had no impact on the enzymatic hydrolysis with or without autohydrolysis. The ink was still detachable after autohydrolysis, as measured by hyperwashing. Compared to untreated newspaper, either mechanical refining or non-ionic surfactant (Tween 80) improved sugar conversion by 10-16% at enzyme dosages of 2 to 8 FPU/g substrate. The combination of both mechanical refining and surfactant resulted in the highest sugar conversions of 46.3%, 56.7% and 64.1% at 2, 4 and 8 FPU/g enzyme dosage, respectively. Oxidative (oxygen at 100 ℃) pretreatment slightly increased sugar conversion of newspaper whereas alkaline (NaOH at 160 ℃) and GL (Na_2CO_3 and Na_2S at 160 ℃) pretreatments had no effect or decreased the sugar conversion. The results of the pretreatments indicate that higher pretreatment temperatures have a negative impact on subsequent enzyme hydrolysis.

Keywords: Newspaper, autohydrolysis, pretreatments, enzymatic hydrolysis, refining, accessible pore volume.

Introduction

Among all kinds of feedstock for bioethanol production, lignocellulosic biomass presents advantages due to low-cost, widespread availability, environmental friendliness and sustainability (Kumar et al., 2008; Solomon et al., 2007). Waste paper, among various other biomass materials, presents a unique opportunity for the production of bioethanol. The shortening of paper fibers during recycling decreases the quality thus waste paper are usually converted into lower grade paper. It has been reported that the maximum ratio of paper-to-paper recycling is 65% (Ikeda et al., 2006). This means an unavoidable portion of cellulose fines are either collected, dried and burnt for process energy, or applied to fields as soil amendment (Duff & Murray, 1996). This portion contains underutilized carbohydrate fraction which can be enzymatically converted into fermentable sugars for ethanol production. In this case, waste paper, compared to other lignocellulosic biomass, presents both environmental and financial benefits (Chen et al., 2014).

Lignocellulosic biomass generally needs to be pretreated to soften the biomass, open the cell structure and make the cellulose fraction more accessible to enzyme digestion. Present pretreatment processes are primarily chemically catalyzed (Hamelinck et al., 2005). Pretreatments such as dilute acid, alkaline, ammonia fiber explosion (AFEX), steam explosion, organic solvent, carbon dioxide explosion etc. have been applied to prepare cellulosic biomass for enzymatic hydrolysis (Alizadeh et al., 2005; Kaar et al., 1998; Kaar & Holtzapple, 2000; Kurakake et al., 2001; Pan et al., 2006; Zheng et al., 1998). Mechanical

pretreatments have also been found to be effective and complementary to chemical pretreatments (Chen et al., 2012; Jones et al., 2013).

Newspaper, among waste paper materials, is rich in cellulose (40-55%), hemicellulose (25-40%), but has a relatively high lignin content (18-30%) compared to other lignocellulosic waste materials (Sun & Cheng, 2002). Extensive researches have been performed towards developing enzymatic hydrolysis processes on conversion of newspaper to ethanol in the last two decades (Duff et al., 1995; Kim et al., 2007; Kim & Chun, 2004a; Kim & Moon, 2003; Lee et al., 2010a; Saxena et al., 1992; Wu & Ju, 1998; Xin et al., 2010). Non-ionic surfactants such as Tween and sodium dodecyl sulfate (SDS) were reported to enhance the enzymatic hydrolysis of newspaper (Kim et al., 2007; Xin et al., 2010). Tween 80 showed ~7% higher digestibility than Tween 20. In another study (Lee et al., 2010a), organosolv pretreatment using high boiling solvent- ethylene glycol was studied on newspaper bioconversion. A glucan conversion of 94% was achieved with the optimum conditions (2% sulfuric acid at 150 ℃ for 15 min) identified in that study. Ammonia fiber explosion (AFEX) and ammonia treatment were found to have marginal improvement on the enzymatic hydrolysis of newspaper (Wang et al., 2012). Ammonia-H_2O_2 was reported to be very efficient on newspaper pretreatment, thereby greatly increasing the susceptibility to enzymatic digestion (Kim & Moon, 2003). In addition, oxidative lime using $Ca(OH)_2$ and O_2 was also found to be efficient. A sugar yield improvement from 33% to 78% at 7.5 FPU/g cellulase (Celluclast 1.5L) dose was achieved (Wang et al., 2012).

As mentioned above, chemical associated pretreatments with/without high temperature have been utilized prior to enzymatic hydrolysis of newspaper, and high enzyme dosage (mostly more than 10 FPU per oven dry g of substrate) was utilized to achieve ideal sugar yields. Anecdotally, it is believed from conference presentations and discussions with enzyme producers, that enzyme dosages in the range of 4-5 FPU/g can be economically attractive (i.e., enzyme cost in the range of $0.50 per gallon of ethanol) (Chen et al., 2014).

Because newspaper has already been chemically and/or physically pretreated during the paper making process, it does not require the extensive pretreatment as required for woody and herbaceous biomass (Kim & Moon, 2003; Xin et al., 2010). As a result, a proposal of utilizing relatively mild autohydrolysis as a pretreatment to treat newspaper followed by enzymatic hydrolysis has been created. Autohydrolysis provides a chemical free/corrosion limited, generally waste free, environmental friendly and low-cost pretreatment technology for fermentable sugar production from lignocelluosic materials (Garrote et al., 1999; Lee et al., 2009). The main sugar component in lignocellulose depolymerized and solubilized in autohydrolysis is xylan (Dekker & Wallis, 1983; Saska & Ozer, 1995). This is due to the catalysis of hydronium ions from water ionization and from in situ-generated acid (such as acetic, uronic and phenolic acids) that cleaves the heterocyclic ether bonds of the xylan backbone and depolymerizing it into xylooligomers and xylose (Garrote et al., 1999).

Although autohydrolysis of various lignocellulosic biomass for ethanol production has been extensively studied (Dekker & Wallis, 1983; Garrote et al., 1999; Grethlein & Converse, 1991; Öhgren et al., 2007; Vázquez et al., 2005), very limited research has been carried out

on the autohydrolysis of newspaper for bioethanol production. In addition, some papers reported that ink had no effect on enzymatic hydrolysis (Duff et al., 1995; Rivers & Emert, 1988; Spano et al., 1976). On the other hand, the negative influence of ink on enzyme digestibility was emphasized in some studies (Kim et al., 2006; Kuhad et al., 2010). Therefore, another objective of this study was to examine the effect of flexo ink, one of the most commonly used newspaper inks, on enzymatic hydrolysis of newspaper. In this work, the effect of autohydrolysis, oxygen, alkaline and green liquor (GL) pretreatments on newspaper were investigated. Surfactant addition and mechanical refining were also evaluated in terms of their effects on newspaper saccharification. The objectives of this study are to identify the limitations and inhibitions involved in bioconverting newspaper to fermentable sugar and to explore treatments to overcome such recalcitrance.

Materials and methods

Materials

Newspaper (issued in January, 2011) and the same unprinted roll-ends were obtained from The Raleigh News & Observer. For the samples subjected to hyperwashing and enzymatic hydrolysis, the newspaper with moisture content of 7% was soaked and repulped using a Tappi disintegrator for 15,000 revolutions (5 min). The pulp was then centrifuged, fluffed and stored in a cold room with sealed plastic zip-lock bags for at least 24 h prior to moisture content determination for enzymatic hydrolysis. For the samples subjected to autohydrolysis, newspaper was shredded into 0.5 cm ×5 cm strips and stored in an air tight plastic bag prior to pretreatment.

Deinking

Disintegrated newspaper (either before or after autohydrolysis) was subjected to a hyperwashing step to analyze the amount of non-detached or redeposited ink. Hyperwashing was conducted in a washing trough with a 200 mesh (74 microns) filter screen using abundant tap water for 10 min. Effective residual ink concentration (ERIC) was measured using a Technidyne Color Touch 2 ISO Model (Technidyne Corporation, New Albany, Indiana, USA) as per the Tappi standard method, T567 pm-97 (TAPPI, 1997). The technique measures reflectance at a 950 nm infrared wavelength from a paper sample over a black back (Jordan & Popson, 1999). An ink detachment parameter (InkD) was calculated to determine the percentage of eliminated ink by the hyperwashing step according to the ERIC values of original newsprint handsheets and of pretreated newsprint handsheets.

Ink detachment parameter (Ink_D) (Pèlach et al., 2003):

$$Ink_D = \frac{ERIC_O - ERIC_P}{ERIC_O} \times 100\%$$

The detachable ink removal (%) determines the % of the defined detachable ink that is removed:

$$\text{Detachable ink removal (\%)} = \frac{ERIC_O - ERIC_P}{ERIC_O - ERIC_u} \times 100\%$$

- $ERIC_O$: ERIC of original newsprint handsheets (ppm);
- $ERIC_P$: ERIC of pretreated newsprint handsheets (ppm).
- $ERIC_u$: ERIC of unprinted newsprint handsheets (ppm).

Note that $ERIC_o$ consists of the freshly applied ink to the newsprint as well as any ink coming with the recycled fibers in the newsprint. Note that $ERIC_u$ is the ink that exists in the newspaper due to residual ink that was contained in recycled fiber; this ink is assumed to be non-detachable since it was not removed in a prior deinking operation.

Pretreatments

Four pretreatment methods were evaluated in this study: autohydrolysis, oxygen, alkaline and green liquor (GL) pretreatments. All the pretreatments were carried out in a 1.5 L stainless steel rotating bomb digester (Thermcraft, Winston-Salem, NC, USA).

Autohydrolysis was carried out with 100 oven dry (OD) g newspaper samples per bomb digester and loaded with 6 times (by weight) of deionized water at 160 ℃ for two retention times-30 min and 60 min. Another set of autohydrolysis experiments were conducted at 180 ℃ for 60 min. For some conditions, 3% or 5% (v/w) acetic acid based on the oven-dry newsprint were loaded to supplement autohydrolysis.

Oxygen pretreatment was carried out with two sodium hydroxide concentrations of 3% and 4% on OD weight of newspaper. The oxygen pressure was set to 100 psig at 100 ℃ for 1 h and the newspaper consistency was 10%.

Sodium hydroxide was used in the alkaline pretreatment at 4% and 6% charges on oven dry weight of newspaper. The pretreatment conditions were 160 ℃ for 1 h with 10% consistency based on oven dry weight of newspaper.

The Green liquor (GL) solution was prepared by mixing sodium carbonate and sodium sulfide with a sulfidity (Na_2S/(NaOH +Na_2S) all as Na_2O) of 25%, which is commonly used in most kraft mills in the United States. The Total Titratable Alkali (TTA) (NaOH +Na_2S+Na_2CO_3 all as Na_2O) charge on the OD weight of newspaper as Na_2O was 4%. The GL pretreatment conditions were set to 160 ℃ for 1 h with 10% consistency based on OD newspaper.

After pretreatment, bomb digesters were cooled in water bath. Pretreated samples were then filtered through cheese cloth, and the 1st filtrates were collected for further determination of pH and sugar content. After the 1st filtration, samples were washed thoroughly and collected for future experiments including handsheet making, post-pretreatment (i.e. hyperwashing) and enzymatic hydrolysis. For chemical composition analysis, the 1st filtrates were subjected to a post acid hydrolysis by adding 72% (w/w) H_2SO_4 to a H_2SO_4 concentration of 4% (w/w). The samples were then incubated in an autoclave for 1 h at 121 ℃. The acid hydrolyzed samples were neutralized by calcium carbonate prior to sugar analysis.

Refining

The newspaper was subjected to 2,500 to 10,000 revolutions of refining in a PFI mill (Hamjern Maskin A/S, Hamar, Norway) using 30 g of OD pulp at 10% consistency, following Tappi procedure T 248 cm-85 (TAPPI, 1993). The pulps were then filtered through cheese cloth. After filtration, the pulps were fluffed, evenly divided, and sealed in plastic zip-lock bags in a cold room until needed.

Enzymatic hydrolysis

Enzymatic hydrolysis was carried out with a consistency of 5% (w/v) at 50 ℃ for 48 h and 180 rpm in an environmental incubator shaker (New Brunswick Scientific, Edison, NJ, USA). An amount of 50 mM of acetate buffer (pH 4.8) with 0.3% (w/v) sodium azide was used. Dry matter content of the treated solid samples and raw materials were determined by an infrared moisture analyzer (Mettler LJ16, Greifensee, Switzerland). Cellulase complex from Trichoderma reesei (NS50013), β-glucosidase from Aspergillus niger (NS50010) and hemicellulase (NS50014) preparations were supplied by Novozymes A/S (Bagsværd, Denmark). The ratio of cellulase:glucosidase:hemicellulase was 1:0.3:0.3. The activity of the cellulase (NS50013) was determined to be 80.5 Filter Paper Unit (FPU)/mL according to methods described by Ghose (Ghose, 1987). Cellulase enzymatic loadings of 2, 4, 8 and 20 FPU/g substrate were evaluated. In some cases, the enzymatic hydrolysis was supplemented with a non-ionic surfactant - sorbitan polyoxyethylene monooleate (Tween 80) at a concentration of 0.4% (v/v) to evaluate its effect on the enzymatic hydrolysis of newspaper.

Specific Surface Areas (SSA) and total pore volume by N_2-BET

Specific surface areas (SSA) of freeze-dried newspaper fibers were measured by a nitrogen adsorption, BET method (Gemini VII 2390, Micrometrics, Atlanta, Georgia, USA). Adsorption was performed with an evacuation rate of 300 mmHg/min, evacuation time of 1 minute, equilibration time of 5 seconds, and relative pressures of 0.05, 0.11, 0.18, 0.24, and 0.30. The total pore volume at the single point p/p0=0.5 on the adsorption curve was used.

Sugar analysis

Compositional analysis of all the treated materials were conducted using the NREL Standard Procedure (Sluiter et al., 2004). Sugar concentrations from enzymatic hydrolysate and filtrates from autohydrolysis were determined using a high-performance liquid chromatography (HPLC) system (Agilent 1200, Agilent, Santa Clara, CA, USA) equipped with a refractive index detector on a Pb-loaded cation exchange column of Shodex Sugar SP0810 (8x300 mm, Showa Denko, Tokyo, Japan). All HPLC samples were filtered through 0.45 μm HV filters (Millipore, Bedford, MA, USA), and a volume of 20 μL was injected. The mobile phase for the column was Milli-Q water at a flow rate of 0.5 mL/min. The system was equipped with a deashing refill cartridge (Bio-Rad 125-0118, Bio-Rad, Hercules, CA, USA). Sugar conversion and sugar recovery were calculated as follows.

$$\text{Sugar conversion} = \frac{Sugar\ released\ (g) \times 0.9}{Carbohydrate\ content\ in\ the\ treated\ material\ (g)} \times 100$$

$$\text{Sugar recovery} = \frac{Sugar\ released\ (g) \times 0.9}{Carbohydrate\ content\ in\ the\ untreated\ material\ (g)} \times 100$$

Newspaper yield from autohydrolysis was calculated based on the starting material (od weight). Separate sugar reductions were calculated based on the sugar analysis of the prehydrolysate liquor from autohydrolysis relative to the sugar in the untreated newspaper samples.

Surface lignin content by XPS

Surface lignin content of newspaper was measured by X-ray Photoelectron Spectroscopy (XPS). The newspaper samples (untreated and pretreated) were first made into handsheets

per Tappi T205(TAPPI, 2002) without pressing. Square samples of ~ 1.1 cm ×1.1 cm area were then cut from the sheets and extracted using acetone. The extraction lasted for 8 hr using a 250 mL soxhlet with a condensate drip rate of ~ 2 drops/second, aiming to remove unexpected extractable impurities from the paper sheets. The newspaper was then subjected to surface lignin measurement by x-ray photoelectron spectroscopy (XPS) with a Mg-Kα excitation (1254 eV) and a PHOIBIS 150 hemispherical analyzer (SPECS, Berlin, Germany) at 10-10 mbar pressure. Energy calibration was established by referencing to adventitious carbon (C1s line at 285.0 eV binding energy). Surface carbon and oxygen content were analyzed to calculate the surface lignin content based on Gray et al.'s method (Gray et al., 2010).Bleached softwood sample with no lignin was handled with the same procedure and used as a reference in the calculation.

Accessible pore volume by DSC

The accessible pore volume, interpreted by the freezing bound water (FBW), was measured by differential scanning calorimetry (DSC) Q100 (TA Instruments, New Castle, DE) and analyzed based on Park's work (Park et al., 2006), with some modifications. Freezing point depression expresses the phenomenon that water in small pores is frozen at low temperature. The depressed freezing temperature has a reciprocal relationship with pore diameters. Therefore, the accessible pore volume can be determined by calculating the accumulation of freezing bound water in different diameters of pores, based on the Gibbs-Thomson equation. In this work, the pulps with consistency of ~35% were sealed in a hermetic aluminum pan and frozen under -80 ℃ for 10 min. The temperature was then raised

to -40 ℃ to determine the sensible heat of the wet fibers without melting. Several heating steps were then conducted from -40, -30, -20, -15, -10, -6, -4, -2, -1.5, -1.1, -0.8, -0.5, -0.2 to -0.1 ℃ with an increasing temperature rate of 1 ℃/min and maintained isothermally at each target temperature until the heat flow returned to the baseline (this required trials for pulps treated differently). The freezing bound water in pore sizes of 1.3 up to 396 nm were calculated based on the integration of each heat-adsorbing segment described above.

Results and discussion

Composition of raw newspaper

The newspaper tested in this study consisted of 75.5% carbohydrate - 58.3% glucan, 8.31% xylan, 0.59% galactan and 8.89% arabinan & mannan, 0.66% acid soluble lignin, 16.0% klason lignin, 2.70% ash and 0.57% extractives. The composition of the newspaper in this study had cellulose, hemicellulose and lignin contents within the range of those from other studies, Table 5.1. Also included in Table 5.1 are values of hardwood and softwood.

Table 5.1 Average biomass composition of untreated newspaper vs. typical lignocelluloses

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Newspaper	58.0	17.7	16.7
Newspaper[a]	60.3	16.4	12.4
Newspaper[b]	53.3	26.3	11.2
Newspaper[c]	40-55	25-40	18-30
Softwood[c]	45-50	25-35	25-35
Hardwood[c]	40-55	24-40	18-25

Source: [a] (Lee et al., 2010a), [b] (Xin et al., 2010) and [c] (Sun & Cheng, 2002).

Effect of ink and surfactant on enzymatic hydrolysis

Different pretreatments were conducted to compare their sugar conversion during enzymatic hydrolysis with original printed newspaper. As shown in Fig. 5.1, original newspaper and hyperwashed newspaper achieved similar sugar conversions at all enzyme dosages. The ERIC values (Table 5.2) of these two substrates show that hyperwashing removed 53.6% of the total ink from the original newspaper, corresponding to 81.2% of the detachable ink removal, since the paper contained non detachable inks from recycled fibers in the paper contributing to 228.3 ppm ERIC. The slight amount of residual ink in unprinted newspaper may be due to the fact that this newspaper is made partially or entirely from recycled paper with non-detachable ink. Negative enzyme inhibition of detachable ink was not observed on sugar conversion based on time-dependent enzymatic hydrolysis (4 FPU/g enzyme dose) data over a period of 108 hours, Fig. 5.2. This is in agreement with the untreated and hyperwashed newspaper having similar enzymatic hydrolysis results in Fig. 5.1.

The effect of surfactant, especially Tween 80 on newspaper saccharification has been studied by several researchers. Sugar conversion improvement of 10-35% was reported depending on enzyme type and dosage, as well as Tween 80 concentrations (Castanon & Wilke, 1981; Wu & Ju, 1998; Xin et al., 2010). Improvements of sugar conversion with the addition of 0.4% (v/v) Tween 80 were also observed herein, Fig.5.1. When considering hyperwashed newspaper, Tween 80 in hydrolysis improved sugar conversion by 7.3%, 8.0% and 6% (all by subtraction) at 2, 4 and 8 FPU/g enzyme dosages.

In addition, enzyme digestibility of newspaper was increased by lab scale mechanical refining, Fig. 5.1. Refining shortened and fibrillated the fibers and created higher accessible surface area for enzyme digestion (Hubbe et al., 2007). Refined newspaper achieved the same sugar conversion compared to the newspaper with Tween 80 addition. Compared with the original newspaper at various enzyme doses, improvement of sugar conversion was from 10% to 16% with refining and/or surfactant usage at all dosages of enzyme studied. This improvement of sugar conversion with surfactant addition agrees with the results in Xin et al.'s study (Xin et al., 2010). By applying surfactant, non-productive binding of cellulase to lignocellulose is reduced by blocking the hydrophobic sites of fiber surfaces (Berlin et al., 2006). Also, surfactant was reported to stabilize enzyme and positively affect enzyme-substrate interactions in order to improve enzyme digestibility (Kim et al., 2006). The combination of both refining and surfactant resulted in the highest sugar conversions, 46.3%, 56.7% and 64.1% at 2, 4 and 8 FPU/g enzyme dosage, respectively.

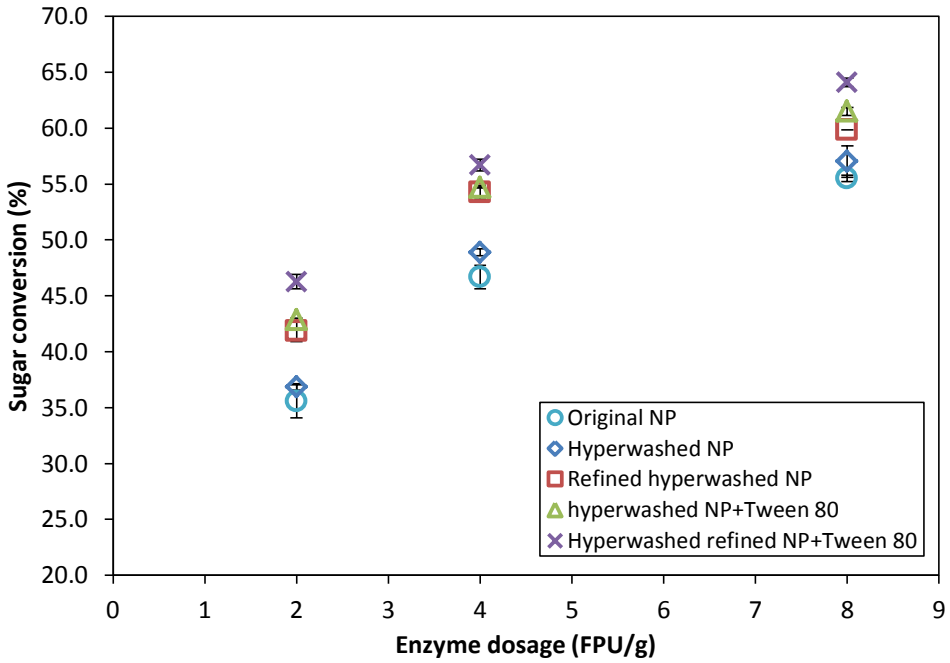

Fig. 5.1 Sugar conversion of newspaper with different pretreatments at 2, 4, and 8 FPU/g enzyme dosage for 48h. Each data point is the average of two replicates; error bars depict ± 1 standard error of the mean

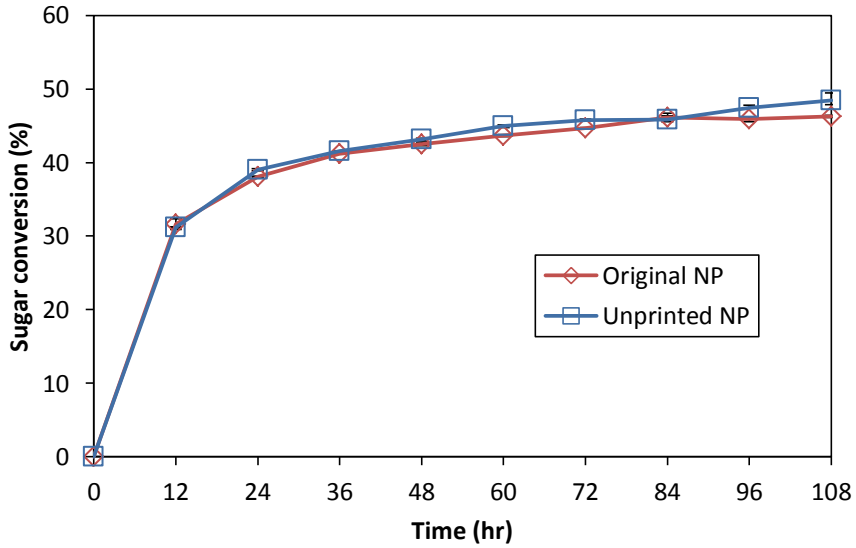

Fig. 5.2 Sugar conversion of original/printed newspaper and unprinted newspaper at 4 FPU/g cellulase dosage. Each data point is the average of two replicates; error bars depict ± 1 standard error of the mean

ERIC values of unprinted/printed newspaper and newspaper with hyperwashing step before or after autohydrolysis were measured, Table 5.2. Hyperwashing before autohydrolysis removed 53.6% of total ink or 81.2% of detachable ink, assuming the ink in the unprinted newspaper is non-detachable. Ink was not removed during autohydrolysis pretreatment, which is a high temperature water treatment process. Handsheets were made from fiber with modest washing and filtration after autohydrolysis and the ERIC values remained the same as the original newspaper. Therefore, the flexo ink was not solubilized or removed during high temperature pretreatment. It was reported that ink becomes stickier during high temperature treatment and deposits on fibers and becomes difficult to remove (Kim & Chun, 2004b). However, hyperwashing after autohydrolysis removed almost the same amount of ink compared to hyperwashing alone, indicating that the flexo ink after high temperature treatment is still detachable.

Table 5.2 ERIC values of newspaper with different treatments

Samples	ERIC (ppm)	Ink detachment parameter (InkD) (%)	Detachable ink removal (%)
Unprinted NP	228.3	/	/
Original NP	672.2	/	/
Hyperwashed NP	311.8	53.6	81.2
160 ℃ 30 min	662.7	1.4	2.1
160 ℃ 60 min	670.1	0.3	0.5
160 ℃ 30 min+ hyperwashing	332.5	50.5	76.5
160 ℃ 60 min+ hyperwashing	363.5	45.9	69.5

Autohydrolysis

Autohydrolysis pretreatment is a hydrothermal process that cooks lignocellulosic materials under high pressure steam at a temperature range of 130-260 ℃ for several seconds

to a few minutes/hours (Lee et al., 2010b). Optimal hemicellulose solubilization and hydrolysis can be achieved by either high temperature and short retention time or lower temperature and longer retention time. It was reported that lower temperature and longer residence time are more favorable (Sun & Cheng, 2002). Autohydrolysis depolymerized the major part of the hemicellulose from the solid material. During this pretreatment, extractives from the solid phase, ash and proteins are solubilized. In addition, partial dissolution of lignin takes place, as well as generation of by-products including furfural and 5-hydroxy-2-methylfurfural (HMF) from sugars (Lee et al., 2009; Pu et al., 2011; Taherzadeh & Karimi, 2007).

In general, the sugar recovery of original, hyperwashed and unprinted newspaper showed that longer retention time solubilized more oligo-sugars, and most sugars generated in filtrates existed as oligomers, Table 5.3. At 160 ℃ and 180 ℃, the main oligo-sugars in filtrates were hemicellulose with only trace amounts of glucose detected. Addition of 3% or 5% acetic acid accelerated dissolution of sugars, and this may be due to the heterocyclic ether bonds of the xylan backbone, which were cleaved to give compounds of a lower polymerization degree via the catalysis of acetic acid (from cleavage of the acetyl groups of the hemicellulose or from supplementation) (Vázquez et al., 2005).

Table 5.3 Reaction time, yield, pH of filtrate, sugars in autohydrolysis filtrate and sugar recovered in autohydrolysis

Conditions	Time (min)	Yield (%)	pH of filtrate	Sugar in filtrate (g)[a]			Sugar recovered (%)[e]
				G[b]	H[c]	T[d]	
Original NP	30	94.1	4.76	0.05	0.41	0.46	0.61
				0.25	2.47	2.72	3.59
	60	93.6	4.50	0.61	0.38	0.99	1.31
				0.92	1.98	2.9	3.83
Hyperwashed NP	30	93.1	4.16	0.06	0.45	0.51	0.68
				0.26	2.28	2.54	3.36
	60	91.4	3.76	0.09	0.56	0.65	0.86
				0.33	2.75	3.08	4.07
Unprinted NP	30	95.0	5.34	0.05	0.29	0.34	0.45
				0.28	1.69	1.97	2.60
	60	95.3	5.28	0.06	0.34	0.40	0.53
				0.30	1.93	2.23	2.95
Unprinted NP+3% AA[f]	60	88.2	3.47	0.20	0.82	1.01	1.34
				0.67	3.61	4.28	5.65
Unprinted NP+5% AA	60	87.2	3.26	0.28	0.99	1.26	1.67
				0.82	4.38	5.20	6.86
Unprinted NP 180 ℃	60	87.8	4.12	0.02	0.26	0.28	0.37
				0.32	1.81	2.13	2.81

Note: expect the "Unprinted NP 180 °C, all the other samples were treated at 160 °C.
[a] Sugar (g) in autohydrolysis filtrate is based on 100 g raw newsprint and sugar recovery is based on total sugar in the raw material. Sugar data before and after 4% H_2SO_4 hydrolysis is listed for each condition.
[b] G: glucan.
[c] H: xylan and other oligo-sugars.
[d] T: total oligo-sugars dissolved in filtrate.
[e] Average % sugar recovered in filtrate based on carbohydrate content in starting materials.
[f] AA: acetic acid.

The yield of the (defined as the % of OD weight obtained after treatments) decreased with time, temperature and acetic acid addition. The compositions of original newspaper and autohydrolyzed newspaper are listed in Table 5.4. In general, autohydrolysis had very little impact on the composition of the residual solids. Few variations were observed on total

carbohydrate and lignin content. The main sugar loss was observed in xylan. Hyperwashing removed about half the amount of ash content.

Table 5.4 Compositions of newspaper and autohydrolyzed newspaper

Substrate	Total Carb (%)	Glu (%)	Xyl (%)	Gal (%)	Ara+ Man (%)	Ash (%)	Acid Soluble Lignin (%)	Klason lignin (%)	Extractives (%)	Mass Balance (%)
Original NP	75.7	58.3	8.3	0.6	8.9	4.71	0.7	16.0	0.57	98.0
Original NP 160℃ 30min	73.7	58.7	4.4	0.52	10.1	4.64	0.54	17.0	N/D	95.9
Original NP 160℃ 60min	74.4	59.3	4.2	0.50	10.4	4.35	0.54	16.9	N/D	96.3
Hyperwashed NP 160 °C 30min	79.4	62.3	5.2	0.77	11.2	2.42	0.50	15.7	N/D	98.0
Hyperwashed NP 160 °C 60min	77.2	61.8	4.8	0.72	9.9	2.30	0.50	15.8	N/D	95.8
Unprinted NP 160 °C 30 min	76.7	59.9	5.2	0.98	10.7	4.43	0.52	16.6	N/D	98.2
Unprinted NP 160 °C 60 min	76.3	59.4	5.0	1.17	10.7	4.31	0.48	16.4	N/D	97.5
Unprinted NP 3% AA 160 °C 60min	77.1	61.8	4.4	0.90	10.1	3.33	0.45	16.8	N/D	97.7
Unprinted NP 5% AA 160 °C 60min	77.4	62.2	4.4	1.12	9.7	2.53	0.44	17.2	N/D	97.6
Unprinted NP 180 °C 60 min	81.2	64.6	2.7	1.4	10.5	4.01	0.48	17.1	N/D	102.7

In this study, sugar conversion of newspaper after autohydrolysis were decreased compared to the control groups, newspaper without pretreatment, Fig. 5.3. The sugar recovery was also decreased since the solubilization of carbohydrate in autohydrolysis was taken as a loss of potential sugar. Even with acetic acid supplementation, enzyme digestibility was not improved, although more hemicellulose was recovered in the filtrate. The sugar conversion of newspaper with autohydrolysis pretreatment plus hyperwashing was still lower than the original newspaper, indicating that a decrease of sugar conversion after autohydrolysis was not due to any effects that the high temperature had to change the composition or distribution of ink in the fibers. In addition, autohydrolysis at higher

temperature (180 ℃) had a greater negative effect on enzymatic hydrolysis compared to autohydrolysis at 160 ℃. When considering that the autohydrolysis had very little effect on composition, the decreases in enzymatic hydrolysis are likely to originate from structural changes.

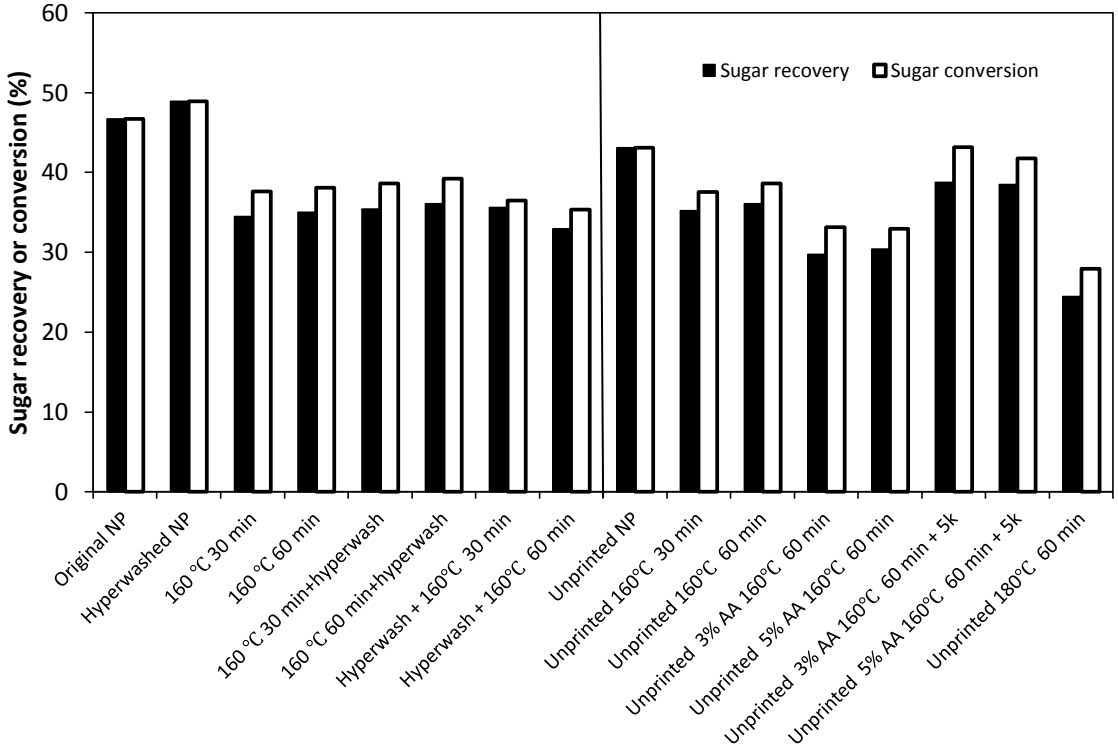

Fig. 5.3 Sugar conversion and sugar recovery of newspaper at 4 FPU/g enzyme dosage under different treatments. Note: the vertical line separates printed newspaper and unprinted newspaper under different treatments. 5k: 5000 revolutions of refining

Higher enzyme dosage was attempted and reconfirmed that autohydrolysis imposed negative influence on enzymatic hydrolysis, Table 5.5. Even at 20 FPU/g enzyme dose, sugar conversion was decreased from 64% to 59% with autohydrolysis. The conversions of all types of sugar were decreased.

Table 5.5 Sugar conversion of unprinted newspaper at 20 FPU/g enzyme dosage with and without autohydrolysis

Pretreatment Conditions	Carbohydrate conversion (%)	Glucan conversion (%)	Xylan conversion (%)	Galactan conversion (%)	Ara+Man conversion (%)
Unprinted NP	64.5	64.1	98.1	75.1	47.5
Unprinted NP 160 °C 30 min	59.2	57.7	83.9	38.6	47.3
Unprinted NP 160 °C 60 min	59.1	57.5	79.7	49.1	46.5

Autohydrolysis of hardwoods and non-woody biomass has been shown to increase the enzymatic conversion of the residual solids (Ertas et al., 2014; Lee et al., 2009; Roman íet al., 2011). Newsprint is produced mainly from softwood. This was confirmed by the average fiber length and bordered pits of the newspaper fibers (Usta, 2005), Fig. 5.4. Hardwood is generally 20-40 μm in width, while softwood is 30-50 μm in width (Smook & Kocurek, 1982). The average width of the newspaper fiber in this study was measured to be 44 μm by sampling multiple fibers (40 observations) under the microscope. The structure of the newspaper fiber is mechanically pulped and liberated fibers with most of the lignin remaining after pulping and existing on the surface of the fibers as parts of the middle lamella. This difference in morphology between mechanical pulp and the original wood may also play a role in the different responses to autohydrolysis.

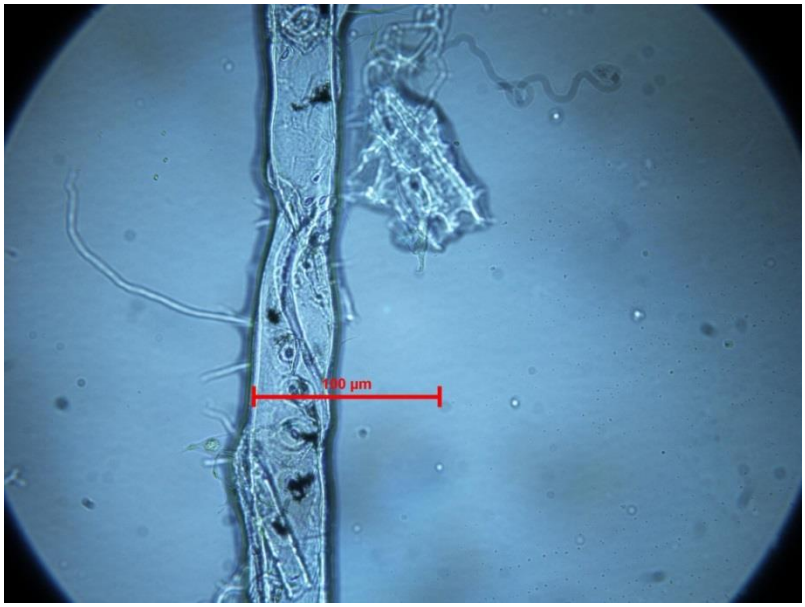

Fig. 5.4 Microscopic image of a representative newspaper softwood fiber, the length scale indicates 100 µm

Autohydrolysis is a pretreatment method which predominantly affects hemicelluloses (Heitz et al., 1991). It was also reported that lignin transformation and relocation to the surface was observed for wheat straw during autohydrolysis (Kristensen et al., 2008). To examine the possibility of lignin relocation for the pretreated newspaper, three separate samples, unprinted NP, unprinted NP with autohydrolysis at 160 ℃ for 60 min and unprinted NP with autohydrolysis augmented with 5% acetic acid at 160 ℃ for 60 min, were selected for surface lignin measurement by XPS, Table 5.6. The corrected molecular ratio of oxygen to carbon $(N_o/N_c)_c$ can be estimated from the measured value by the following equation (Gray et al., 2010):

$$\frac{1}{(N_O / N_C)_{pulp,corrected}} = \left[\frac{2.88 * (N_O / N_C)_{cellulose,measured} - 0.833}{1.88 * (N_O / N_C)_{cellulose,measured}}\right] * \left[\frac{1}{(N_O / N_C)_{pulp,measured}} + 1\right] - 1$$

Using this equation and the O/C ratio for cellulose of 0.833 and for lignin of 0.335, the surface lignin can be estimated (Gray et al., 2010). Compared to the control group, unprinted newspaper with 0.202 segment mole fraction and 22% weight fraction of surface lignin content, newspaper after autohydrolysis with or without acetic acid did not significantly change the estimated surface lignin content. Hence, lignin relocation is not in evidence with these measurements and as such cannot be identified as the source negative impact of autohydrolysis on sugar conversion of newspaper.

Table 5.6 Surface lignin content of newspaper samples

Samples	$(No/Nc)_m$	$(No/Nc)_c$	Segment mole fraction of lignin (S_L)	Weight fraction of lignin (W_L)
Unprinted NP	0.758	0.686	0.202	22%
Unprinted NP 160 ℃ 60 min	0.766	0.693	0.192	21%
Unprinted NP 5% AA 160 ℃ 60 min	0.744	0.674	0.222	24%

Note: $(No/Nc)_m$: measured molecule ratio of oxygen and carbon; $(No/Nc)_c$: corrected molecule ratio of oxygen and carbon.

Accessible pore volume to enzymes has also been used to explain differences in enzyme digestibility of biomass (Yu et al., 2011). To investigate the effect of accessible pore volume on newspaper saccharification, freezing bound water measurements on the control and autohydrolysis treated newspaper samples were performed, Fig. 5.5. It is shown that after treating newspaper at 160 ℃ for 60 min with only water, total pore volume was dramatically reduced, mainly due to the collapse of pores with a diameter larger than 30 nm. The same reduction in pore volumes were determined for autohydrolysis conditions at higher

temperatures or with acetic acid addition, Fig. 5.5. The pore collapse observed is consistent with the lower sugar conversion experienced after autohydrolysis.

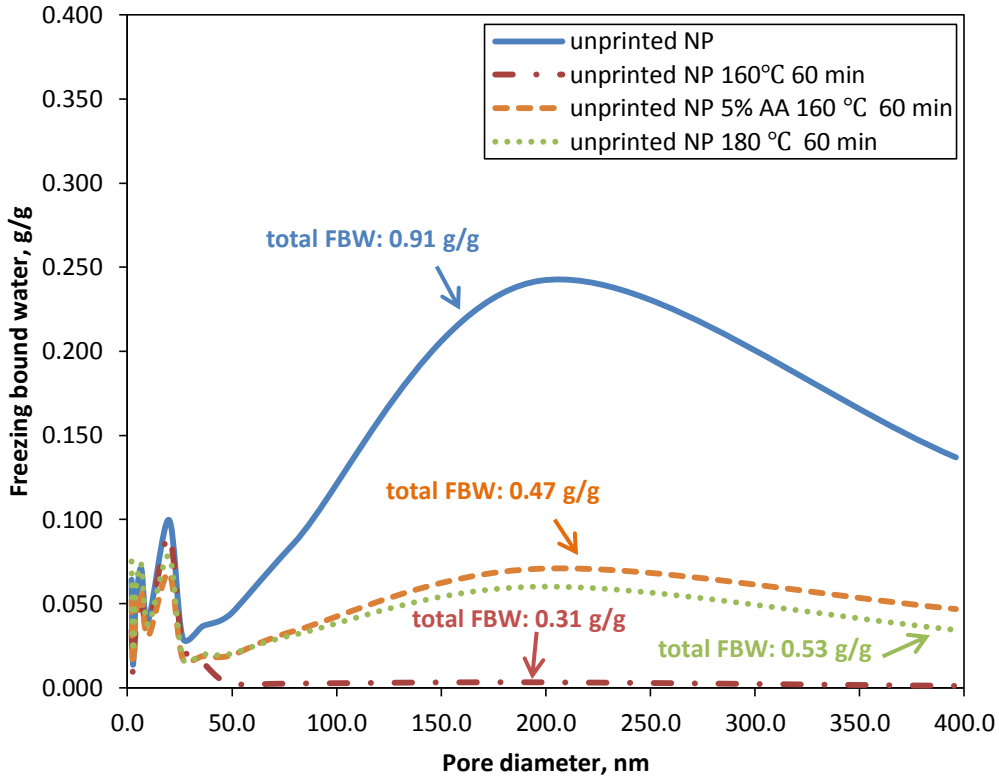

Fig. 5.5 Pore diameter distribution and total accessible pore volume expressed by freezing bound water (FBW) of unprinted and autohydrolyzed newspaper

Effect of refining on enzymatic hydrolysis

It was expected that mechanical refining would result in increases in internal delamination and external fibrillation, increasing accessible fiber areas to enzyme and thus increasing the enzymatic hydrolysis efficiency. This result has been shown previously for wood pulps such as chemically pulped and bleached recycled copy paper fibers (Chen et al., 2012). The unprinted newspaper was refined by a laboratory PFI mill with 0, 2,500, 5,000,

7,500 and 10,000 revolutions. Escalation of refining intensity resulted in an increase of specific surface area (SSA) and total pore volume, Fig. 5.6. Refining of unprinted newspaper fiber resulted in considerably higher surface areas, which has been reported as a determinant of the rate and degree of hydrolysis of the substrate (Nazhad et al., 1995). The sugar conversion was improved with refining from 43% to 56% by 10,000 revolutions of PFI refining.

BET nitrogen adsorption measures the specific surface area (SSA) of materials in the dry state. Although SSA from BET method does not represent the actual SSA of newspaper fiber in the wet state, it should be reflective of the wet state. It is known that mechanical fibers do not shrink on drying to a large extent relative to chemically pulped fibers. This is due to the lignin content of the mechanical fibers. Also, the newsprint in this study had already been refined and dried prior to the rewetting during the autohydrolysis/enzyme hydrolysis and subsequent drying. It is known that the shrinkage of fiber occurs to the greatest extent on the first drying cycle and less on subsequent cycles. For both of these reasons, the newsprint studied with the BET method herein is assumed to have some but minimal shrinkage prior to the BET measurement. The increase in SSA and total pore volume are consistent with the increases in sugar conversion, as it is expected that refining increases the accessible areas of the substrate for enzyme hydrolysis.

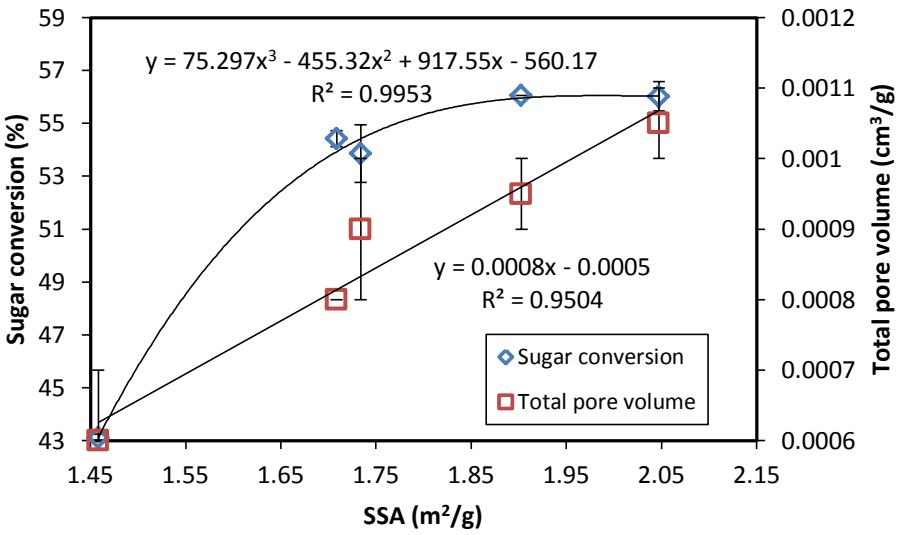

Fig. 5.6 Sugar conversion of unprinted newspaper with different refining severity versus the corresponding specific surface area (SSA) and total pore volume as measured by N_2-BET. Each data point is the average of two replicates; error bars depict ± 1 standard error of the mean

Fiber coarseness, an important parameter in papermaking, is defined as the mass of fibers per unit length. Fibers with high coarseness have thick cell walls, which would be expected to have lower enzyme hydrolysis rates than fibers with very thin cell walls (with higher SSA). Refining is expected to reduce coarseness by breaking off parts of the fiber wall, leaving lower mass in the fiber wall, Table 5.7. Also fine material should contribute to lower coarseness of the pulp overall. Decreases in coarseness correlated with increased sugar conversion. However, this is just a correlation, decreased fiber length and increased fines also correlated with increases in sugar conversion upon refining.

Table 5.7 Refined fiber properties by different refining intensity

Refining (revolutions)	% Fines	Mean length (mm)	Coarseness (mg/m)
0	8.33±0.27	1.32±0.03	0.145±0.001
2500	9.21±0.08	1.09±0.007	0.135±0.001
5000	9.16±0.13	1.09±0.002	0.131±0.002
7500	9.01±0.26	1.17±0.002	0.133±0.005
10000	9.32±0.09	1.08±0.006	0.110±0.002

Impact of oxygen, alkaline and green liquor (GL) pretreatments on enzymatic hydrolysis

As discussed above, autohydrolysis at 160 ℃ with 30 and 60 min did not improve the enzymatic hydrolysis of newspaper. Even with acetic acid addition, this pretreatment was not effective on newspaper. Thus, other pretreatments were attempted such as oxygen, alkaline and green liquor (GL) pretreatments. These pretreatments are expected to remove lignin and thus improve enzyme accessibility and efficiency.

Oxygen pretreatment (100 ℃) achieved 97.4% yield with 3% NaOH and 93.3% yield with 4% NaOH when compared to the starting material using unprinted newspaper. The oxygen pretreatments did not appreciably change the chemical composition of the newspaper, Table 5.8. Green liquor (160 ℃) and alkaline pretreatments (160 ℃) with 4% or 6% NaOH partially removed lignin from the starting newspaper and pretreatment yields of 86%, 82% and 80% were obtained, respectively.

Table 5.8 Pretreatment yields and compositions (based on treated newspaper) of oxygen, alkaline and green liquor (GL) pretreated newspaper

Substrate	Temperature (℃)	Yield (%)	Total Carb (%)	Glu (%)	Xyl (%)	Gal (%)	Ara+Man (%)	Ash (%)	Acid Soluble Lignin (%)	Klason lignin (%)
Unprinted NP	/	/	75.7	58.3	8.3	0.6	8.89	2.70	0.66	16.0
Oxygen (3% NaOH)	100 °C	97.4	77.9	57.4	7.8	1.3	11.4	3.69	0.66	17.2
Oxygen (4% NaOH)	100 °C	93.3	81.8	59.7	8.5	2.0	11.6	3.48	0.66	16.2
Green liquor	160 °C	86.3	85.1	64.3	6.9	2.0	11.9	3.92	0.78	13.0
Alkaline (4% NaOH)	160 °C	81.7	86.1	65.9	7.3	1.8	11.1	4.15	0.75	13.1
Alkaline (6% NaOH)	160 °C	80.3	88.3	68.9	7.7	1.4	10.3	3.89	0.76	11.7

Compared to sugar conversion of unprinted newspaper, only oxygen pretreatment marginally improved enzymatic digestibility from 43% to 46% and from 55% to 60% at 4 and 8 FPU/g enzyme dosage, Fig. 5.7. This is interesting since this pretreatment did not remove lignin as did the green liquor and alkaline pretreatments and thus might not be expected to be better. Further, this pretreatment was the only pretreatment occurring at a relatively low temperature, 100 ℃. This suggests that the elevated pretreatment temperatures in autohydrolysis, green liquor and alkaline pretreatments may be the cause of the negative effect. The NaOH dose of 3% or 4% in oxygen pretreatment made little difference in terms of sugar conversions.

Green liquor is an effective pretreatment for hardwood in terms of enzymatic hydrolysis (Jin et al., 2010). However, green liquor alone is not efficient to improve the sugar conversion of softwood (Wu et al., 2010). Although newspaper has been through the pulping and paper making process, which presumably makes fiber more amenable for enzyme digestion, green liquor was not able to improve sugar conversion because newspaper is

mainly made of softwood in this case. Lastly, alkaline pretreatments at 4% and 6% NaOH showed lower sugar conversion than the untreated NP, where the two conditions present similar performance in enzymatic hydrolysis.

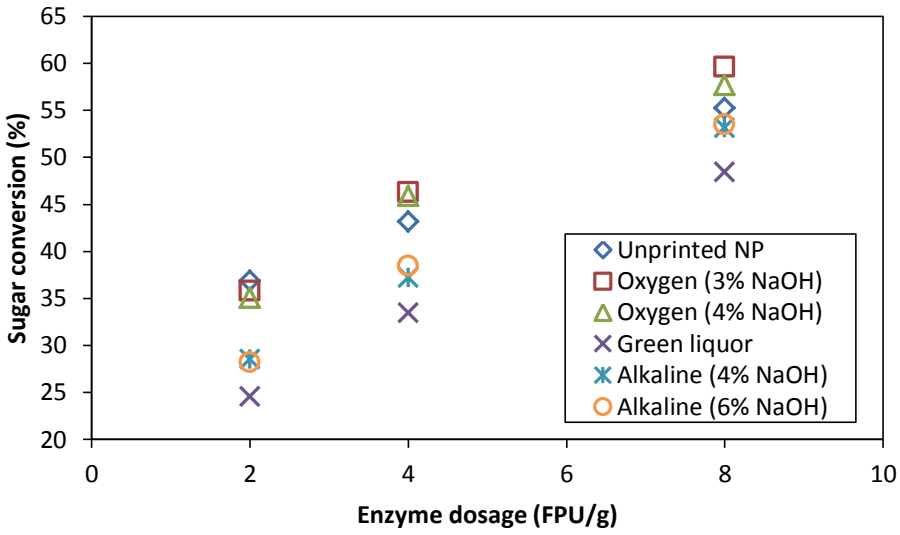

Fig. 5.7 Sugar conversion of unprinted newspaper, oxygen, green liquor (GL) and alkaline pretreated newspaper. Each data point is the average of two replicates; the average of the standard error of the mean for these results is 0.4

Conclusions

Mechanical refining and non-ionic surfactant Tween 80 positively influenced enzymatic hydrolysis of newspaper. Escalation of the refining intensity resulted in the increase of SSA and total pore volume, which positively correlated with sugar conversion. However, refining also caused increases in fines and decreases in coarseness that correlated with sugar conversions. Autohydrolysis at 160 and 180 ℃ had a negative effect on enzyme hydrolysis. The same trend was found for autohydrolysis with acid addition. Oxygen, alkaline and green liquor (GL) pretreatments showed that only the oxidative approach marginally increased

sugar conversion; the alkaline and GL pretreatments had a negative effect. Interestingly, the oxygen pretreatment was the only pretreatment performed at a lower temperature (100 ℃). There seems to be a disadvantage of increasing the temperature in pretreatments since the lowest sugar conversion was achieved with the highest autohydrolysis temperature.

Acknowledgments

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Chapter 6 Fundamental Study of Lignin Removal by Different Pretreatments on Enzymatic Hydrolysis of Softwood

Abstract

It is widely acknowledged that softwood is more recalcitrant to enzymatic hydrolysis than hardwood. Since softwood is an indispensable element in woody biomass to ethanol, it is necessary to develop an efficient pretreatment process and understand its influence on the structure of softwood. Four types of pulp were generated by kraft pulping (KP76), sodium chlorite (KP133-D), oxygen (KP133-O) and high consistency ozone (KP133-HZ) delignification with similar bulk lignin content (11.3%-13.7%). Sugar conversions during enzymatic hydrolysis were the highest for KP133-D, 89.4% with an enzyme dose of 5 FPU/g pulp and 97.2% at 10 FPU/g. while kraft pulp KP76 had the lowest. Neither accessible pore volume nor water retention value (WRV) was found to correlate with enzymatic hydrolysis. Lignin isolated from softwood samples with approximately equal bulk lignin contents showed that lignin hydrophobicity had a linear relationship with sugar conversions, which are 42.2% for KP76 pulp, 89.4% for KP133-D pulp, 63.2% for KP133-O pulp and 72.8% for KP133-HZ pulp at an enzyme dose of 5 FPU/g of substrate. Phenolic hydroxyl groups in lignin were identified as an inhibitor to enzymatic hydrolysis. However, the total hydroxyl groups and aliphatic hydroxyl groups in softwood lignin favored sugar conversion in enzymatic hydrolysis.

Keywords: softwood, pretreatment, lignin, lignin hydrophobicity, hydroxyl groups.

Introduction

Softwoods are the dominant lignocellulosic feedstocks in the Northern hemisphere and viewed to be attractive and sustainable for ethanol fuel production. However, lignocellulosic biomass, especially softwood, is recalcitrant to biological degradation due to the rigid and compact structure as well as its chemical composition (Mooney et al., 1998; Pan et al., 2005; Wu et al., 2010). The recalcitrance comes from the cellulose crystalline structure (Mittal et al., 2011) and its degree of polymerization (Pan et al., 2008), hemicellulose (heterogeneity and physical blockage) (Yoshida et al., 2008), lignin (physical blockage, lignin-carbohydrate complex (LCC), nonproductive adsorption of enzymes and deactivation of enzymes by soluble lignin (Nakagame et al., 2010). Physical properties such as particle size, surface area and pore volume are considered to be factors as well (Dasari & Berson, 2007; Koo et al., 2011; Yu et al., 2011). Due to the more heterogeneous nature of lignocellulosic biomass, the relationship of these recalcitrance and enzyme digestibility is not completely clear. However, among all the factors, it has been proven that lignin is the dominant factor affecting biomass enzymatic reactivity in the overall hydrolysis (Chang & Holtzapple, 2000; Laureano-Perez et al., 2005).

Lignin is the third most abundant natural polymer present on earth after cellulose and hemicellulose, and the most abundant non-carbohydrate component composed of aromatic moieties in lignocellulosic biomass. The lignin content varies in lignocellulosic biomass and usually in the range of 18-35% for wood, and 10-30% for grass biomass such as corn stover, sugarcane bagasse, wheat straw, rice straw and switchgrass (Buranov & Mazza, 2008; Zhao

et al., 2012). Softwood in general has approximately 10% more lignin than hardwood (Lamlom & Savidge, 2003). Structurally, lignin is an amorphous polymer consisting of phenylpropane units. Softwood is mainly composed of guaiacyl lignin (G), a polymerization product of coniferyl alcohol, while hardwood lignin is typically guaiacyl-syringyl lignin (S), a copolymer of coniferyl and sinapyl alcohols. Small amounts of p-hydroxyphenyl moietites (H) are found in both hardwood and softwood (Santos et al., 2012). Grass biomass typically contain all three lignin units (G, S, H) in significant amounts with different ratios (Buranov & Mazza, 2008).

Since lignin content governs the enzymatic hydrolysis of biomass, reduction of lignin is important for removing the recalcitrant barriers and increasing enzymatic digestibility of cellulose. In the past few decades, many lignin removal strategies have been developed to make the lignocellulosic substrate more susceptible to enzymatic hydrolysis. In this research, kraft pulping, and kraft pulping followed by oxygen, sodium chlorite, low and high consistency ozone delignification techniques were considered.

Kraft pulping is the prevalent chemical pulping process in North America. In kraft cooking, sodium hydroxide and sulfide (known as white liquor in aqueous solution) react with lignin, breaking it into smaller water/alkali-soluble fragments through the cleavage of linkages in phenolic arylpropane units. Generation of free phenolic hydroxyl groups takes place increasing the hydrophilicity of lignin and cleaved lignin fragments (Chakar & Ragauskas, 2004; Johansson & Ljunggren, 1994). Oxygen and sodium chlorite delignification has been used in the pulp and paper industry to remove lignin during

bleaching stages. One of the mechanisms for oxygen delignification is phenolic delignification. In this process, phenolic hydroxyl groups in lignin (generated from kraft pulping) react with alkali and generate phenolate ions that form reactive intermediate phenoxy radicals. Demethoxylation-ring cleavage and degradation to acids or coupling of phenoxy radicals take place with different reactions by phenoxy radicals (Johansson & Ljunggren, 1994). Sodium chlorite delignification in dilute acetic acid solution is selective in the removal of lignin with only trace degradation of polysaccharides (Hubbell & Ragauskas, 2010). Ozone delignification is driven by oxidant and the oxidative species formed during ozonation treatment. It preferably reacts with aromatics and olefinic structures in lignin and both polysaccharides and lignin are attacked by formed hydroxyl radicals, which is the dominant factor affecting the selectivity of ozone treatment (Ek et al., 1989; Eriksson & Gierer, 1985). Stabilizers such as acid media or pyrophosphate are necessary for this process. In terms of ozone consistency, it has been reported that low consistency ozone treatment is more selective to lignin, but with less efficient ozone utilization compared to a high consistency ozone treatment (Brolin et al., 1993).

In this research, softwood by kraft pulping process and subsequent treatments (oxygen, sodium chlorite, low and high consistency ozone delignification) were compared in terms of enzyme digestibility. Pulp generated by these treatments with similar Kappa number or lignin content were studied, aiming to identify the factors causing different enzymatic sugar conversions. An attempt was made to relate various parameters such as pore volume of fiber, lignin distribution and hydrophobicity to enzyme digestibility of softwood.

Materials and Methods

Kraft pulping

Fresh loblolly pine chips were obtained from a mill in the United States and cooked with a stock solution of NaOH and Na_2S in the lab. Four cooking conditions were conducted. H-factors were controlled to 600, 800, 1200 and 1500. Total Titratable Alkali (TTA) as Na_2O on oven dried (OD) wood chips for the four conditions were 14%, 16%, 18% and 19%. Cooking temperatures were performed at 170 ℃ with 25% sulfidity and a liquor-to-wood ratio of 4. At the end of pretreatment, the wood chips were washed with tap water to completely remove residual chemicals and dissolved wood components. They were then refined to pulp using a Bauer 148-2 disk refiner twice with a disk gap of 0.25 mm and 0.05 mm. The pulp was then centrifuged to remove excessive water, fluffed and stored at 4 ℃ until further usage. Kappa numbers were measured on the accepted pulp according to TAPPI T236 cm-85 (TAPPI, 1985). Kraft pulps are named "K-Kappa number" in the following texts.

Pulp Compositional analyses of all the pulps were conducted using the Standard NREL Procedure in three parallel tests (Sluiter et al., 2004). Sugar (glucose, xylose, galactose, arabinose and mannose) analysis was performed by a high-performance liquid chromatography (HPLC) system (Agilent 1200, Agilent, Santa Clara, CA, USA) equipped with a deashing filter (Bio-Rad 125-0118, Bio-Rad, Hercules, CA, USA), a Shodex SP0810 column (8x300 mm, Showa Denko, Tokyo, Japan) and a refractive index detector. The mobile phase for the column was Milli-Q water at a flow rate of 0.5 mL/min at a temperature of 80 ℃.

Post treatments

Oxygen, sodium chlorite, low and high consistency ozone delignification were conducted on kraft pretreated pulps with a Kappa number of 133. Oxygen (named as O) delignification was carried out in a bomb digester with 5% sodium hydroxide at 110 ℃. The reaction length was 1 hour at an oxygen pressure of 100 psia. Sodium chlorite (named as D) delignification was carried out in a 70 ℃ water bath for 15 min. Four levels of sodium chlorite solutions were prepared as 0.5%, 1.0%, 1.5% and 2.0% based on OD pulp. Pulp consistency was controlled at 3% (w/v) with 0.6% (v/v) acetic acid. Ozone delignification was conducted at high (~35%) and low (1%) consistency (w/v) (named as HZ & LZ). Before ozone treatment, the kraft pretreated pulp was diluted to 1% consistency and pH adjusted to 2 using 4N sulfuric acid. The pulps were then centrifuged a consistency of ~35%, fluffed and stored in the cold room overnight at 4 ℃. Moisture content was measure the next day to determine the pulp consistency for HZ treatment and how much pH 2 sulfuric acid solution should be added for the LZ treatment. Pulps of 25 OD g were then transferred into a spherical glass reactor. Ozone was generated from an ozone generator by passing bulk oxygen through 35kV. A mixture of ozone and oxygen was pumped into the reactor with stabilized ozone concentration of 5% (v/v) and a flow rate of 1L/min. The reaction time varies by HZ and LZ treatment to generate 4 levels of HZ pulps and 5 levels of LZ pulps in terms of lignin content. All the post-treated pulps were then washed thoroughly to remove remaining chemicals, centrifuged and stored in the cold room at 4 ℃.

Enzymatic hydrolysis

Cellic® CTec2 (cellulase complex blended with high level of cellulases, beta-glucosidase, and hemicellulase) from Novozymes (Franklinton, NC, USA) was applied for all the enzymatic hydrolysis experiment in this study. Total cellulase activity of 136 Filter Paper Unit (FPU)/mL was confirmed for Cellic® CTec2 using the filter paper assay as described in the International Union of Pure and Applied Chemistry standard method (Ghose, 1987). Enzyme dosage was 5 or 10 FPU/ OD g pulp with a solid content of 5% (w/v) at 50 ℃ for 48 h and 180 rpm in an environmental incubator shaker (New Brunswick Scientific, Edison, NJ, USA). Sugar concentrations (glucose, xylose, galactose, arabinose and mannose) for composition analysis and enzymatic hydrolysis results were quantified using a high-performance liquid chromatography (HPLC) system (Agilent 1200, Agilent, Santa Clara, CA, USA). The HPLC system was equipped with a deashing filter (Bio-Rad 125-0118, Bio-Rad, Hercules, CA, USA) and a Shodex SP0810 column (8x300 mm, Showa Denko, Tokyo, Japan). The mobile phase was Milli-Q water at a flow rate of 0.5 mL/min and temperature of 80 ℃. The sugar conversion was calculated as follows

$$\text{Sugar conversion} = \frac{Sugar\ released\ (g) \times 0.9}{Carbohydrate\ content\ in\ the\ treated\ material\ (g)} \times 100 \quad (1)$$

Accessible pore volume

The accessible pore volume, interpreted by the accumulation of freezing bound water (FBW), was measured by differential scanning calorimetry (DSC) Q100 (TA Instruments, New Castle, DE) and analyzed based on Park's work (Park et al., 2006) with some modifications. Freezing point depression is expressed as the phenomenon when water in

small pores freezes at lower temperatures. The depressed freezing temperature has a reciprocal relationship with pore diameters. Therefore, the accessible pore volume can be determined by calculating the accumulation of freezing bound water in different diameters of pores, based on the Gibbs-Thomson equation. In this work, the pulps with consistency of ~35% were sealed in a hermetic aluminum pan and frozen under -80 ℃ for 10 min. The temperature was then raised to -40 ℃ to determine the sensible heat of the wet fibers without melting. Several heating steps were then conducted from -40, -30, -20, -15, -10, -6, -4, -2, -1.5, -1.1, -0.8, -0.5, -0.2 to -0.1 ℃ with temperature increasing at a rate of 1 ℃/min and maintained isothermally at each target temperature until heat flow returned to baseline (required trials for pulps treated differently). The freezing bound water in pore sizes of 1.3 up to 396 nm were calculated based on the integration of each heat-adsorbing segment described above.

Water retention value (WRV)

WRV was determined using the TAPPI Method (TAPPI, 1981). A fiber mat with a surface density of 1400 g/m ^{2}was required on the crucibles in this procedure. The pulp pad was centrifuged with a centrifugal force (Eppendorf North America, Hauppauge, New York, USA) of 900 rcf (2,400 rpm) for 30 min. The fiber was then removed from the crucibles and placed in a pre-weighed aluminum pan with lid, and the wet weight was recorded. The pan was then oven dried at 105 ℃ overnight. The samples were then cooled in a desiccator for 30 min before weighing the dry weight. The WRV was expressed as the weight of water one unit weight of fiber can retain, i.e.,

$$\text{WRV (g/g)} = \frac{w_{wet} - w_{dried}}{w_{dried}} \times 100 \quad (2)$$

where

w_{wet} is the wet weight of the substrate;

w_{dried} is the oven-dried weight of the substrate.

Lignin distribution

Lignin distribution for the 4 types of pulp was measured using confocal laser scanning microscopy (CLSM). A staining step was required before imaging the samples. Acridine orange (AO) (3,6-Bis(dimethylamino) acridine hydrochloride, Sigma-Aldrich, St. Louis, MO) solution was prepared with deionized water to achieve a concentration of 1.25×10^{-5} M. Approximately 200 mg of sample was placed in a 25 mL glass vial with 20 mL of AO solution. The vial was sealed to avoid light and shaken periodically for 1 h. After staining, the samples were washed by deionized water 3 times. The fiber samples were then placed on a 3 in×1 in glass slide covered with a cover slip and sealed with wax. The measurement was performed using a Carl Zeiss LSM 710 confocal workstation with a C-Apochromat 40x/1.1 water immersion objective lens and an argon laser at 488 nm as excitation wavelength. Image analysis was performed using ZEN lite 2011 image analysis software. Fluorescence emission between 515 and 540 nm was collected as green fluorescence which represent fiber rich in carbohydrate, and fluorescence emission above 590 nm was collected as red fluorescence which represent fiber rich in lignin (Li & Reeve, 2005). Lignin concentration was calculated based on a calibration curve developed with red/green emission shift values and Klason lignin measurements.

Lignin isolation

Softwood samples (KP76, K133-D, K133-O and K133-HZ) with approximately equal bulk lignin contents (11.3%-13.7%) were subjected to lignin isolation. Two lignin isolation procedures were conducted in this study. The first procedure includes two steps of enzymatic hydrolysis, aiming to obtain maximum yield of cellulolytic enzyme lignin (CEL), Fig. 6.1. Softwood pulp was first enzymatically hydrolyzed at 15 FPU/g Ctec2 dosages for 96 h. The whole suspension was then pH adjusted to 3 by 1M HCl in order to precipitate all the lignin. After centrifugation, the solid fraction was washed with a HCl solution 3 times and freeze dried for extraction. Extraction of the solid fraction was conducted using 80% (v/v) 2, 4-dioxane for 24 h, three times. After the first extraction, the solution was separated by centrifugation. The dioxane solution was evaporated by a rotary evaporator under vacuum at 35 ˚C. A few drops of deionized water was added to the residue to remove traces of 2, 4-dioxane, and repeated several times when necessary. The concentrated solution was then carefully transferred into a pre-weighted beaker, freeze dried for 2 days and vacuum dried at 35 ℃ for 1 day to obtain CEL I. The solid fraction was freeze dried and subjected to the second enzymatic hydrolysis at a dose of 15 FPU/g Ctec2 for 24 h. The same procedure for solid/liquid separation and drying method was performed to obtain CEL II.

Since some of the CEL isolated using the procedure showed in Fig. 6.1 were not dissolvable in DMSO-d6 for ^{13}C-NMR analysis due to the high molecular weight lignin extracted by 80% (v/v) 2, 4-dioxane. Further study required another procedure to obtain both CEL and milled wood lignin (MWL), Fig. 6.2. The pulp was first subjected to enzymatic

hydrolysis and extraction with the same methods in the first procedure to obtain CEL except that 96% (v/v) 2, 4-dioxane was used this time for extraction. The enzymatic hydrolysis residue was then ball milled for 3 h at 600 rpm using ZrO_2 bowls and 17 ZrO_2 balls in a planetary ball mill (Pulverisette 7, Fritsch, Germany). During balling milling, 30 min of suspension time was applied for every 30 min of grinding. The wood meal was then extracted with 96% (v/v) 2, 4-dioxane for 12 h at room temperature, twice. After centrifugation, the solid remains were kept for the next phase of extraction and the liquid part was collected. The liquid part was filtered to remove small solid particles and then the solvent removed by a rotary evaporator under vacuum at 35 ˚C. A few drops of deionized water was added to the residue to remove traces of 2, 4-dioxane, and repeated several times when necessary. The lignin was then carefully transferred into a pre-weighted beaker, freeze dried for 2 days and vacuum dried at 35 ℃ for 1 day to ensure complete drying. This portion of lignin is non-purified MWL.

Lignin purification was conducted by dissolving non-purified isolated lignin in 90% acetic acid and precipitated into 10 times of water. The precipitated lignin was separated by centrifugation, and then freeze dried and vacuum dried to obtain purified cellulolytic enzyme lignin (CELp) and purified milled wood lignin (MWLp).

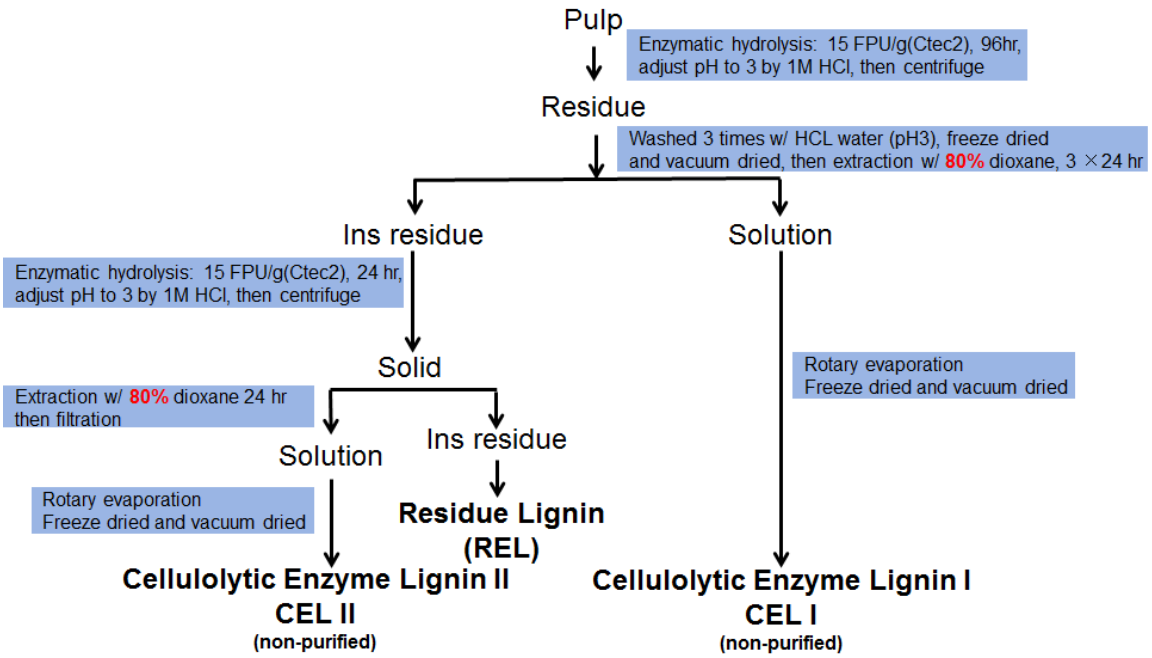

Fig. 6.1 Lignin isolation procedure with two steps of CEL I and CEL II extraction

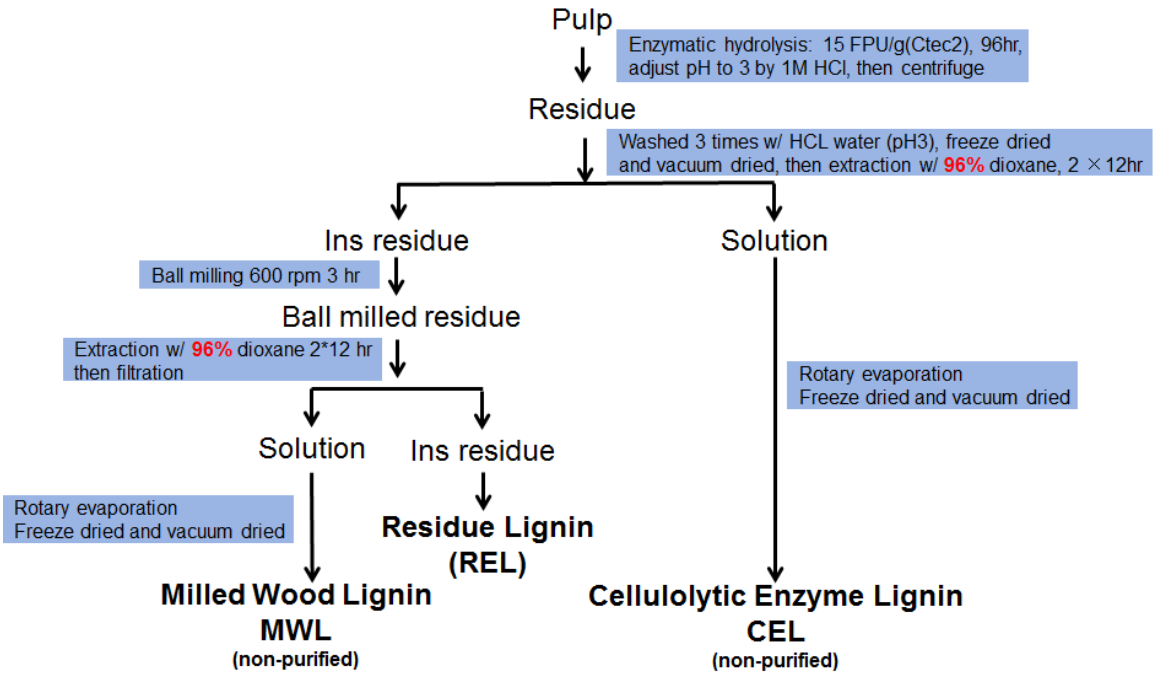

Fig. 6.2 Lignin isolation procedure with CEL and MWL extraction

Composition analysis of isolated lignin

Due to the limited amount of isolated MWL, a modified protocol for sugar analysis was developed. Lignin sample of 20 mg was swollen in 0.75 mL of 72% H_2SO_4 at room temperature for 2 h. The sample was then diluted with 42 mL of deionized water (4% H_2SO_4) and autoclaved at120 ℃ for 1 h. After completion of the reaction, the sample was cooled and filtered to separate Klason (acid insoluble) lignin from the liquid fraction. Acid soluble lignin was measured using the liquid fraction by absorbance at 205 nm in a HP 8453E UV-VIS spectrometer.

Sugar in the lignin was analyzed using the liquid fraction with a high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex ICS-3000, Dionex Corporation, Sunnyvale, CA, USA). The HPAE-PAD was equipped with a CarboPac PA1 (100μeq per 4 x 250 mm) analytical column operated at 18 °C with degassed Milli-Q water (0.22 μm, Millipack express 20, Millipore) and 400 mN sodium hydroxide solution as the mobile phase at a flow rate of 0.3 ml/min, a CarboPac PA1 guard column (4 x 50 mm), a thermostatted autosampler, and a dual pump. A 200 mN sodium hydroxide solution was added to post column for optimal detector sensitivity and baseline stability. Samples injections were run for 70 min. An internal standard (fucose) solution of 10 mg/ml was added to each sample for the determination of monomeric sugars.

^{31}P–NMR analysis

CELp and MWLp were subjected to ^{31}P–NMR analysis. Hydroxyl functional groups in the purified lignins have been identified by a ^{31}P–NMR technique that involves derivatization with the phosphorylating agent 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP) (Argyropoulos et al., 2014). The reaction of TMDP with hydroxyl functional groups is illustrated in Fig. 6.3. TMDP reacts with hydroxyl functional groups to give phosphite products which are resolvable by 31P–NMR into separate regions arising from aliphatic hydroxyl, phenolic, and carboxylic acids groups.

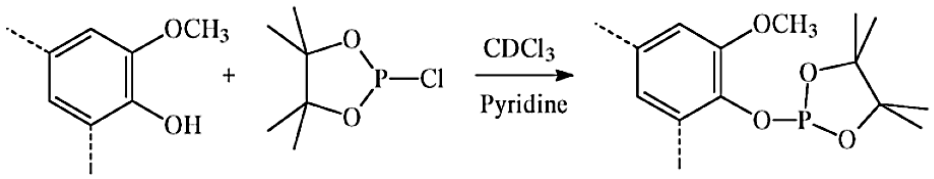

Fig. 6.3 Derivatization of phenolic structures with 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP)

Procedure for sample preparation is as follows. An accurate weight (about 40 mg) of a dried lignin sample was carefully transferred into a 5 mm NMR sample tube. The following chemical solutions were then added into the tube: 500 μL of anhydrous pyridine/$CDCl_3$ mixture (1.6:1, v/v), 200 μL of an endo-N-hydroxy-5-norbornene-2, 3-dicarboximide (e-NHI) solution (serving as an internal standard, 9.23 mg/mL), 50 μL of chromium (III) acetylacetonate solution (serving as a relaxation reagent, 5.6 mg/mL) and 100 μL of the phosphitylating reagent (2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane). The tube was then capped and placed in a water bath with ultrasound to ensure complete dissolution.

Each ^{31}P-NMR acquisition was performed using a Bruker AVANCE 300 MHz spectrometer with a 25 s delay between 90 °pulses. An inverse gated decoupling pulse sequence was used to obtain quantitative spectra. A number of 256 transients were acquired for each sample. The acquisitions were performed at room temperature, using a 62 ppm sweep width (TD = 32,768) and a 4 Hz line broadening. The total acquisition time was about 25 min. Chemical shifts were calibrated relative to the internal standard with signal centered at δ 152.0 ppm. Typical ranges for each hydroxyl functional groups in this quantification method are listed in Table 6.1.

Table 6.1 Chemical shifts of each hydroxyl groups in the ^{31}P-NMR analysis

Chemical Shift, ppm	Assignment
145-150	Aliphatic OH
144.4-140.4	Condensed Phenolic OH
140.4-137	Non condensed Phenolic OH
136.5-133	Carboxyl OH

Lignin films

Multi-layered lignin films were prepared for contact angle analysis. A 0.5 % (w/w) lignin solution was prepared by dissolving purified CEL or MWL in 96% (v/v) 1,4-dioxane until full dissolution. Silicon chips were cut to 1 ×1 cm^2 squares exposed to ultraviolet radiation for 5 min for UV/ozonator prior to film deposition. They were then pre-coated with polystyrene for the purpose of coating uniform lignin films. The lignin solutions were then filtered through a 0.22 μm nylon syringe filter. A few drops of filtered lignin solution were spread on the silicon chip mounted on the spin coater. The lignin film was spin coated at

2000 rpm for 10 s with an acceleration time of 1 s. This process was repeated 4 times to ensure full coverage of the lignin films.

Water contact angle (WCA) analysis

Lignin hydrophobicity was measured by initial water contact angle and dynamic water contact angle with the lignin films using a Phoenix 300 contact angle analyzer (SEO Co. Ltd, Lathes, South Korea) with Image XP software capturing the droplet images. A water drop (5 μl) was manually deposited with a syringe onto the solid. The drop on the substrate was recorded at 2 images per second for 10 s. Images were then captured after 1, 2, 4, 6, 8 10 min to analyze the dynamic contact angle. The contact angle from each image was analyzed using the NIH software ImageJ.

Results and discussion

Effect of pretreatments

Before pretreatment, lignin content of fresh chips (extractive free) was 28.5% and K133 pulp had a lignin content of 21.1%. Four types of pulp were generated by kraft pulping (KP), sodium chlorite (KP133-D), oxygen (KP133-O) and high consistency ozone (KP133-HZ) delignification with similar bulk lignin (Klason + acid soluble lignin) content (11.3%-13.7%). Although their lignin contents are similar, their kappa numbers are different due to the different degrees of oxidation of residual lignin. KP133-D had the highest sugar degradation and acid soluble lignin content, Table 6.2.

Table 6.2 Chemical compositions of the pulp generated by kraft, sodium chlorite, oxygen and high consistency ozone delignification with similar bulk lignin content

Pulp	Kappa #	Total Carb (%)	Glu (%)	Xyl (%)	Gal (%)	Ara+Man (%)	Total lignin (%)	Mass Balance (%)	Klason lignin, g	Acid soluble lignin, %
KP76	76	86.6	69.3	6.9	1.8	8.6	13.0	99.6	12.4	0.7
KP133-D	48	77.5	62.6	6.8	1.5	6.6	13.7	91.2	11.5	2.2
KP133-O	40	84.2	66.2	6.3	1.4	10.2	12.0	96.1	11.2	0.8
KP133-HZ	78	85.3	66.2	6.5	1.6	11.0	11.3	96.7	9.8	1.5

Enzymatic hydrolysis was conducted at enzyme doses of 5 and 10 FPU/g substrate, Table 6.3. At the same enzyme dose and approximately the same bulk lignin content, enzymatic hydrolysis efficiencies of these four types of pulp vary dramatically. Kraft pulp at a Kappa number of 76 achieved only 42.4% sugar conversion at 5 FPU. K133-D achieved the highest sugar conversion – 89.4% followed by K133-HZ at 72.8% and K133-O at 63.2%. Sugar yield is the monomeric sugars in the hydrolysate based on the loaded OD weight of the pulp. Sugar yields for these pulps showed the same trend with sugar conversions.

Table 6.3 Sugar yields and sugar conversions of KP76, KP133-D, KP133-O and KP133-HZ pulp at enzyme doses of 5 and 10 FPU/g substrate

Pulp	Sugar yield, %		Sugar conversion, %	
	5 FPU	10 FPU	5 FPU	10 FPU
KP76	40.7 (1.6)	59.6 (0.5)	42.2 (1.6)	61.8 (0.5)
KP133-D	77.0 (0.3)	87.6 (0.01)	89.4 (0.4)	97.2 (0.01)
KP133-O	59.1 (0.2)	78.7 (0.3)	63.2 (0.3)	84.2 (0.3)
KP133-HZ	69.0 (0.1)	83.8 (0.2)	72.8 (0.1)	88.4 (1.3)

Accessible pore volume and water retention value (WRV)

Water retention value (WRV) is an indicator of accessible surface area and porosity of cellulosic fibers (Luo & Zhu, 2011). Accessible pore volume was measured and expressed by the total freezing bound water (FBW) value, Table 6.4. No correlation was found between sugar conversion with WRV or FBW. However, there is a linear relationship between WRV and FBW, Fig. 6.4, which confirmed the irrelevance between accessible pore volume and enzymatic efficiency. One possible explanation could be that different oxidation mechanisms of lignin removal enable these four types of pulp to have different properties. Pore volume itself, in this particular case, cannot explain the variation in sugar conversions of enzymatic hydrolysis.

Table 6.4 Water retention value (WRV), total freezing bound water (FBW) and sugar conversion (5 FPU/g enzyme dosage) of treated softwood fibers

Pulp	WRV, g/g	FBW, g/g	Sugar conversion, %
KP76	2.02 (0.04)	0.96	42.2
KP133-D	1.66 (0.01)	0.89	89.4
KP133-O	2.04 (0.01)	0.99	63.4
KP133-HZ	1.64 (0.02)	0.75	73.2

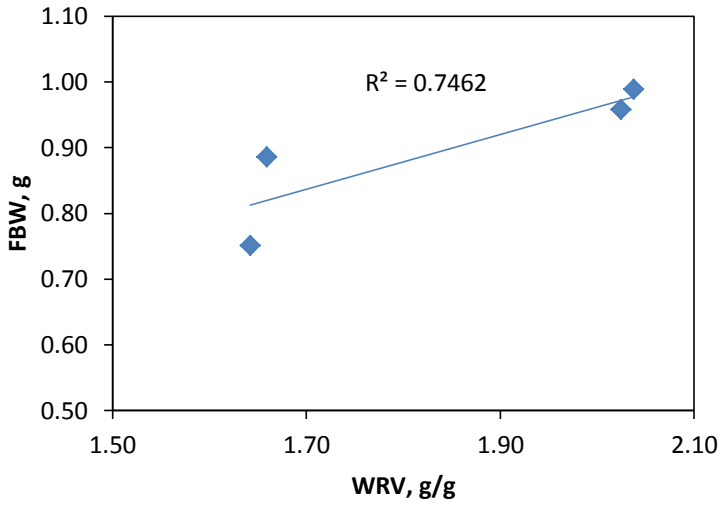

Fig. 6.4 Correlation between water retention value (WRV) and total freezing bound water (FBW)

Lignin distribution

Lignin distribution of the four types of fibers was measured by confocal laser scanning microscopy (CLSM). The red to green emission shift in the images represent the intensity of carbohydrate (green) and lignin (red) in the fiber across the fiber cell wall, Fig. 6.5. The lignin distribution of the fibers was calculated based on the analysis of at least five representative fibers from each treatment. A calibration curve was developed with red/green emission shift values and Klason lignin measurements. K76, KP133-D, KP133-O and KP133-HZ fibers present different lignin distributions, Fig. 6.6. In general, KP76, KP133-D and KP133-O had the highest concentration of lignin on the outer surface of the fiber and it decreased to different extents from the outer to the lumen surface. The lignin concentration is more evenly distributed for KP76 compared to other pulps since kraft cooking is the least severe among all the treatments. Due to the rapid reaction in ozone delignification, the lignin in the KP133-HZ fiber was completely removed from the outer and up to 25% of the lumen

surface. KP133-HZ also had ~20% lignin in the rest of the areas compared to ~10% for other three types of fibers.

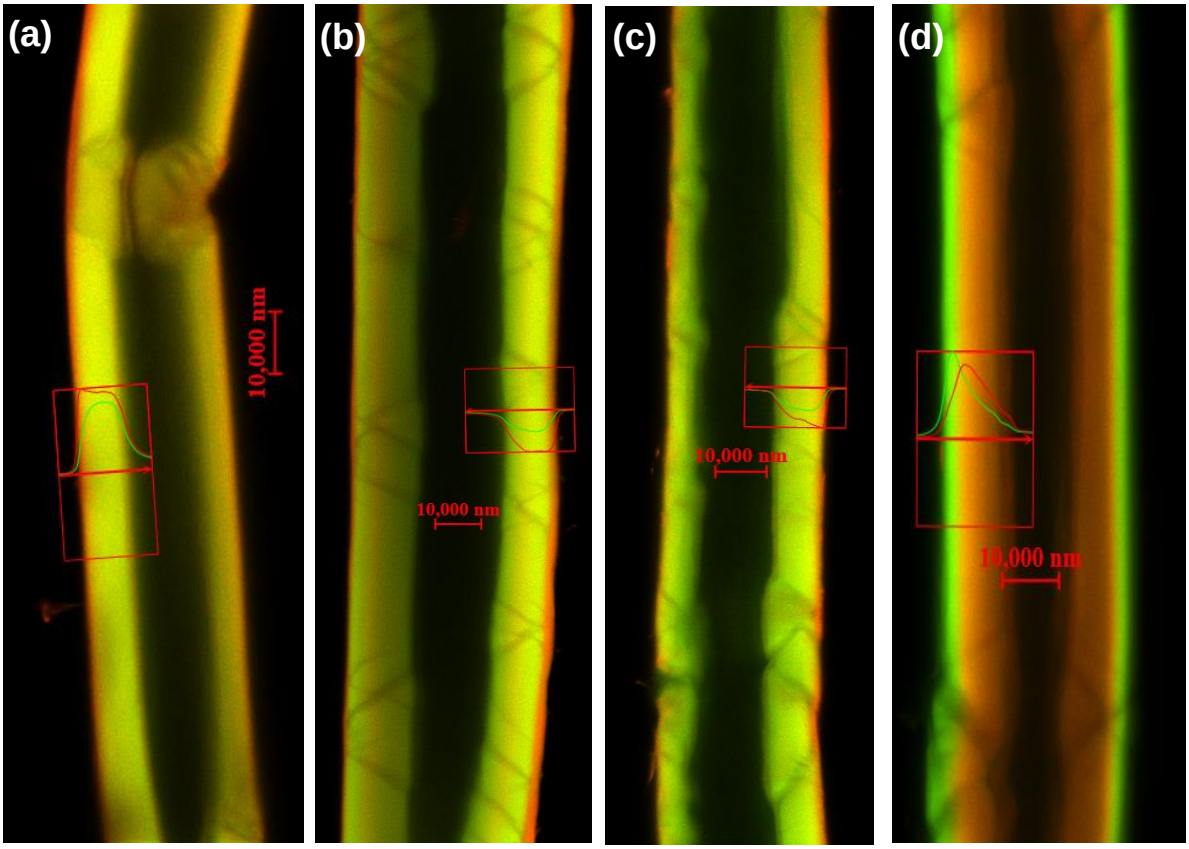

Fig. 6.5 confocal laser scanning microscopy (CLSM) images of the four types of fibers. (a) kraft fiber (KP76); (b) sodium chlorite treated fiber (KP133-D); (c) oxygen treated fiber (KP133-O) and (d) high consistency ozone treated fiber (KP133-HZ)

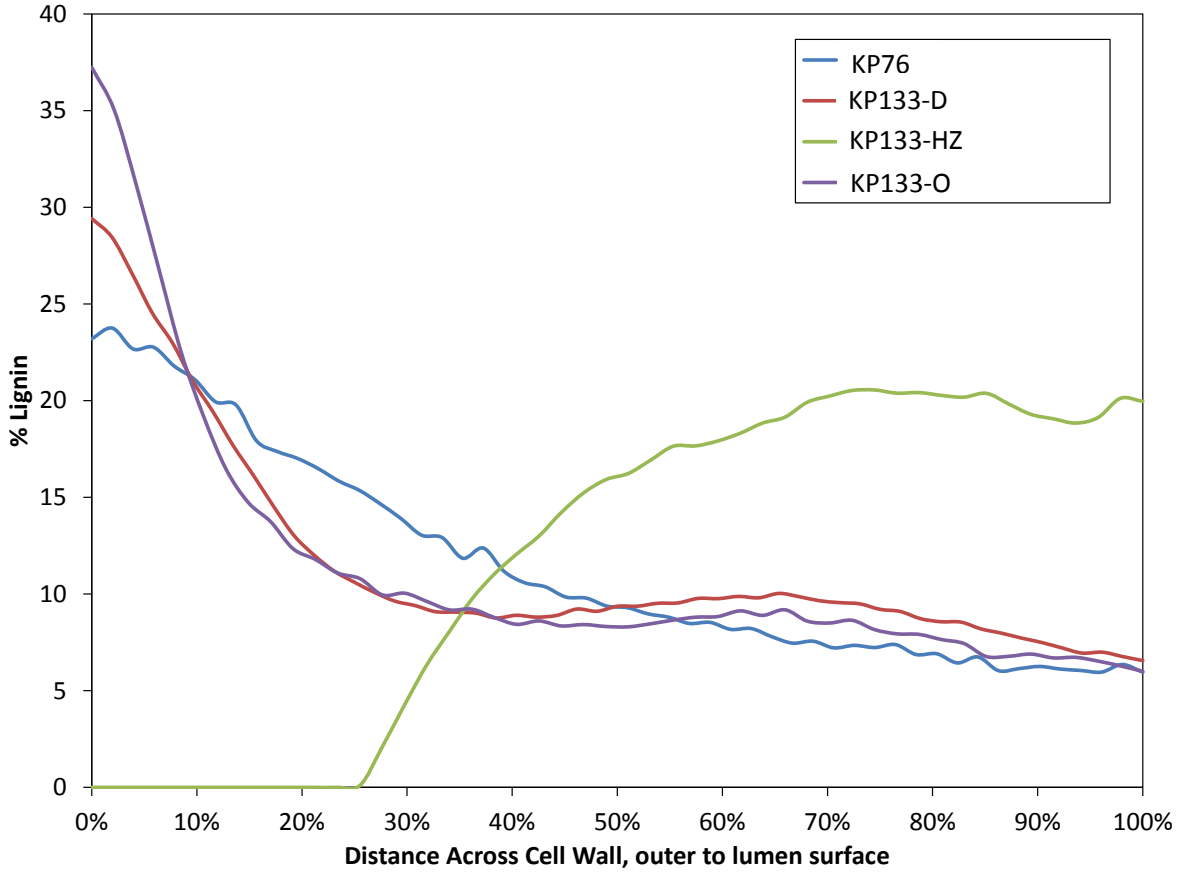

Fig. 6.6 Lignin distribution of KP76, KP133-D, KP133-HZ, KP133-O fibers. X-axis: the normalized distance across the fiber cell wall beginning from the outside surface (0%) to the inside lumen surface (100%); Y-axis: the weight percent of lignin

Lignin isolation

Lignin was isolated from these four types of pulp with the procedure shown in Fig. 6.2. The yields of non-purified CEL and MWL are listed in Table 6.5. KP76 had the highest yield for CEL – 19.1% and KP133-D only achieved 6.3% yield for CEL. This is due to the fact that sodium chlorite is very powerful in terms of lignin removal and residual lignin after sodium chlorite treatment is considered "hard-to-remove" lignin. The yields of MWL were

similar (12%-16%) except for KP133-HZ (28%). The combined yields for both CEL and MWL ranged from 18.1% to 41.5%.

Table 6.5 Kappa number, total lignin content in each type of pulp and the yields of lignin isolation

Pulp	Kappa	Lignin, %	CEL yield, %	MWL yield, %	Combined yield, %
KP76	76	13.0	19.1	14.9	34.0
KP133-D	48	13.7	6.3	11.7	18.1
KP133-O	40	12.0	10.5	15.9	26.5
KP133-HZ	78	11.3	13.7	27.8	41.5

Composition of non-purified CEL and MWL was analyzed by measuring sugar content and back calculating the lignin content, Table 6.6. The reason for using this approach is that our previous attempt on lignin composition analysis was not successful by using the modified NREL Standard Method mentioned in the "Materials and methods" section. The Klason lignin was measured by the weight of the solid after autoclave and the acid soluble lignin by absorbance at 205 nm in a UV-VIS spectrometer. This method worked very well with KP76 lignin. However, the total lignin contents in all the oxidized lignin were underestimated. One reason is that after oxidation, acid soluble lignin was structurally altered and became undetectable at the wavelength of 205 nm.

The crude/non-purified CEL and MWL was further purified to obtain similar sugar content for subsequent experiments. The purification yields for all CEL were below 75%. Except for CEL from KP133-D, which had 23% sugar, the compositions of all the other CELp (purified) did not change too much compared to the corresponding crude CEL. This indicates that the difference between CELp and CELc (crude/non-purified) is mainly due to

molecular weight. During lignin purification using acetic acid, lignin with higher molecular weight precipitated out, while the lignin with lower molecular weight remained soluble. The same trend was also found in MWL purification.

Table 6.6 Compositions of non-purified and purified isolated lignin and purification yields

Lignin		Non-purified		Purification yield, %	Purified	
		Theoretical lignin, %	Sugar, %		Theoretical lignin, %	Sugar, %
KP76	CEL	86.1	13.9	47.3	89.9	10.1
	MWL	94.0	6.0	83.1	93.5	6.5
KP133-D	CEL	76.9	23.1	74.4	88.6	11.4
	MWL	94.7	5.3	42.9	95.6	4.4
KP133-O	CEL	88.1	11.9	46.2	88.1	11.9
	MWL	93.7	6.3	76.9	91.9	8.1
KP133-HZ	CEL	88.2	11.8	51.9	94.7	5.3
	MWL	94.9	5.1	60.1	95.4	4.6

^{31}P–NMR analysis

CELp and MWLp were subjected to ^{31}P–NMR analysis to determine major hydroxyl functional groups. The chemical shifts were well distinguished among the internal standard, aliphatic OH, phenolic OH (condensed and uncondensed) and carboxylic OH, Fig. 6.7.

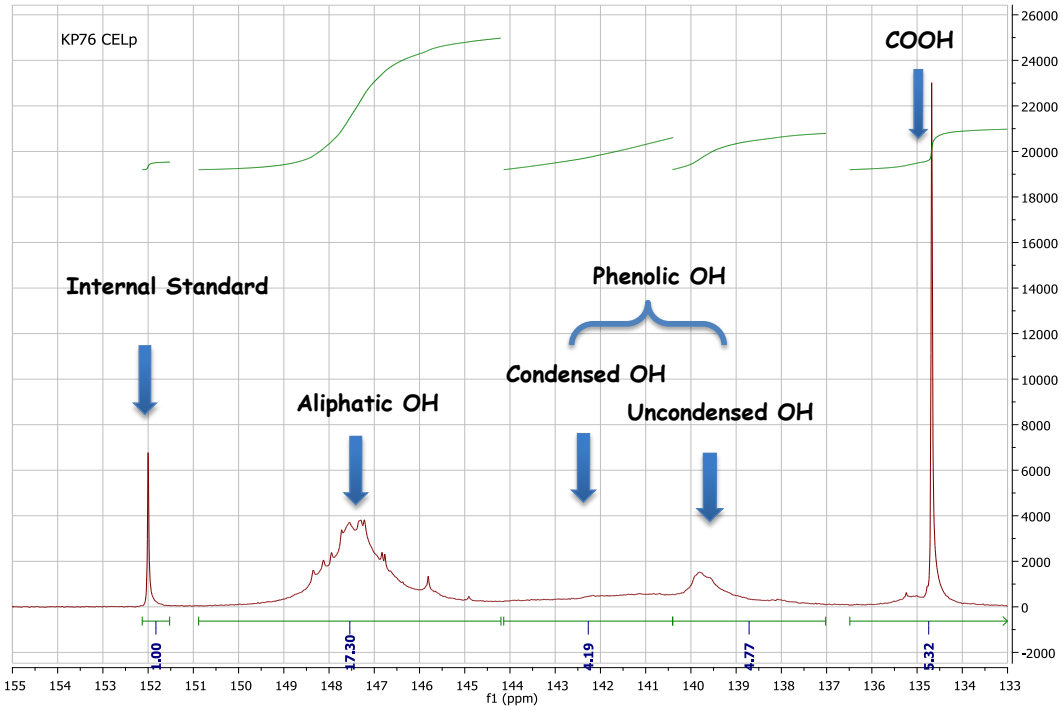

Fig. 6.7 ^{31}P–NMR spectra of KP76 CELp to demonstrate chemical shifts of different hydroxyl groups

From the ^{31}P–NMR spectra, the amount of hydroxyl groups in softwood CEL and MWL with various treatments was calculated and expressed as mmol/g, Table 6.7. Assuming the molecular weight of C9 units for softwood MWL is 185 g/mol (Argyropoulos et al., 2002), the analysis showed that in CELp, phenolic OH was reduced in oxidized lignin compared to CELp in KP76 pulp. This is due to the opening of benzene rings during the oxidation process. CELp in K133-D pulp had the highest total OH groups. For all MWLp, the amounts of each hydroxyl groups were similar. The phenolic OH was increased in MWLp compared to the corresponding CELp. This is due to the fact that the hydrolysis took place during the ball milling and generated more phenolic hydroxyl groups. As a result, CEL is more suitable to study the correlation between lignin and enzymatic hydrolysis efficiency compared to MWL.

Table 6.7 Hydroxyl functional groups of various CELp and MWLp in mmol/g and number of groups/C9

Unit	Functional groups	K76		K133-O		K133-D		K133-HZ	
		CELp	MWLp	CELp	MWLp	CELp	MWLp	CELp	MWLp
mmol/g	Aliphatic OH	4.80	4.46	5.44	4.93	8.24	4.66	4.49	4.38
	Condensed Phenolic OH	1.16	1.33	0.84	1.17	0.74	1.26	1.11	1.11
	Non condensed Phenolic OH	1.32	1.53	0.73	1.10	0.57	1.35	1.23	1.16
	Phenolic OH	2.49	2.86	1.57	2.27	1.31	2.60	2.34	2.27
	Carboxyl OH	1.48	0.70	1.52	1.18	1.58	0.86	1.73	0.91
	Total OH	8.76	8.03	8.53	8.38	11.14	8.12	8.56	7.56
/100 C9	Aliphatic OH	89	83	101	91	152	86	83	81
	Condensed Phenolic OH	22	25	16	22	14	23	21	21
	Non condensed Phenolic OH	24	28	14	20	11	25	23	21
	Phenolic OH	46	53	29	42	24	48	43	42
	Carboxyl OH	27	13	28	22	29	16	32	17
	Total OH	162	149	158	155	206	150	158	140

Hydroxyl groups of lignin contribute to its hydrophobicity. A correlation was identified between hydroxyl groups in purified lignin and sugar conversions, Fig 6.8. Phenolic hydroxyl groups were found to be an inhibitor to enzymatic hydrolysis. It is in agreement with some other studies that claim phenolic hydroxyl groups of lignin had negative impact on enzymatic hydrolysis above certain concentrations (Pan, 2008). Greater amounts of hydroxyl or aliphatic hydroxyl groups in the lignin yielded better enzymatic hydrolysis efficiencies to the corresponding pulp. The same trend was reported in opposite orders regarding enzyme adsorption on lignin versus total OH, aliphatic OH and phenolic OH (Yu et al., 2014).

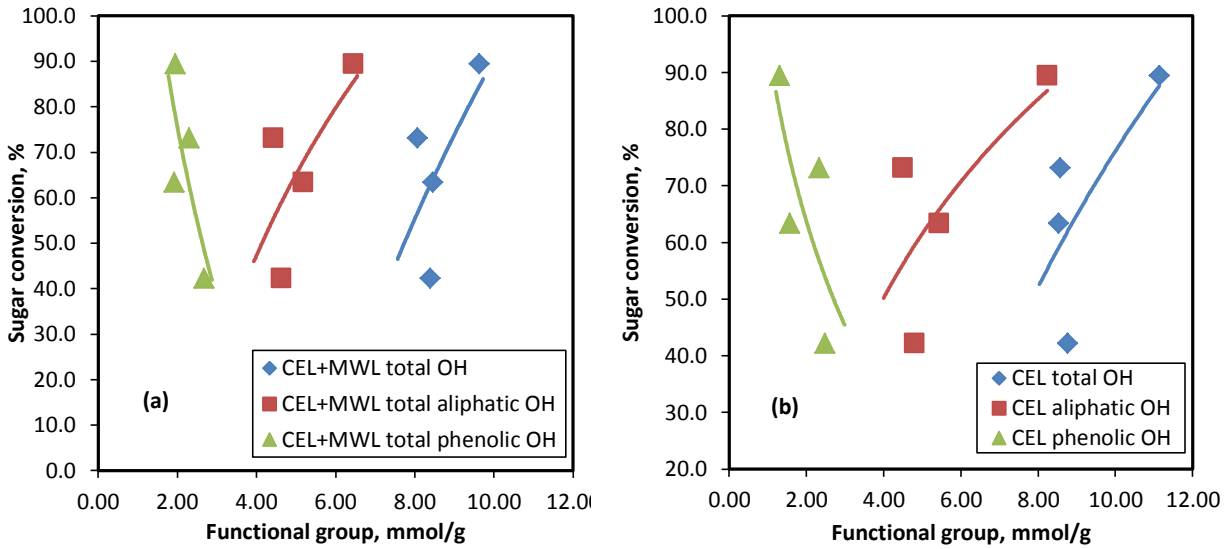

Fig. 6.8 Hydroxyl functional groups in CELp and MWLp vs. sugar conversions

Lignin hydrophobicity

Lignin films were prepared by spin coating lignin-dioxane solutions onto silicon chips. Similar to the trends found in ^{31}P–NMR analysis, initial water contact angle (WCA) of CELp varied from 53.1 ºto 59.0 º, Table 6.8. After ball milling, the hydrophobicity of the lignin (MWLp) was similar due to the hydrolysis reaction occurring during ball milling, which resulted in similar hydroxyl content for all four MWLp.

Table 6.8 Initial water contact angle of purified isolated lignin CELp and MWLp

Lignin		WCA, º
KP76	CELp	59.0
	MWLp	53.8
KP133-O	CELp	56.6
	MWLp	52.6
KP133-D	CELp	53.1
	MWLp	52.4
KP133-HZ	CELp	54.8
	MWLp	53.0

Dynamic water contact angle (DWCA) from 0.5s to 10 min showed that K133-D CELp was the most hydrophilic and KP76 CELp was the most hydrophobic lignin followed by K133-O, KP133-HZ and KP133-D, Fig. 6.9. After ball milling, the dynamic behavior of all MWLp became similar, which was already shown in the hydroxyl group measurement.

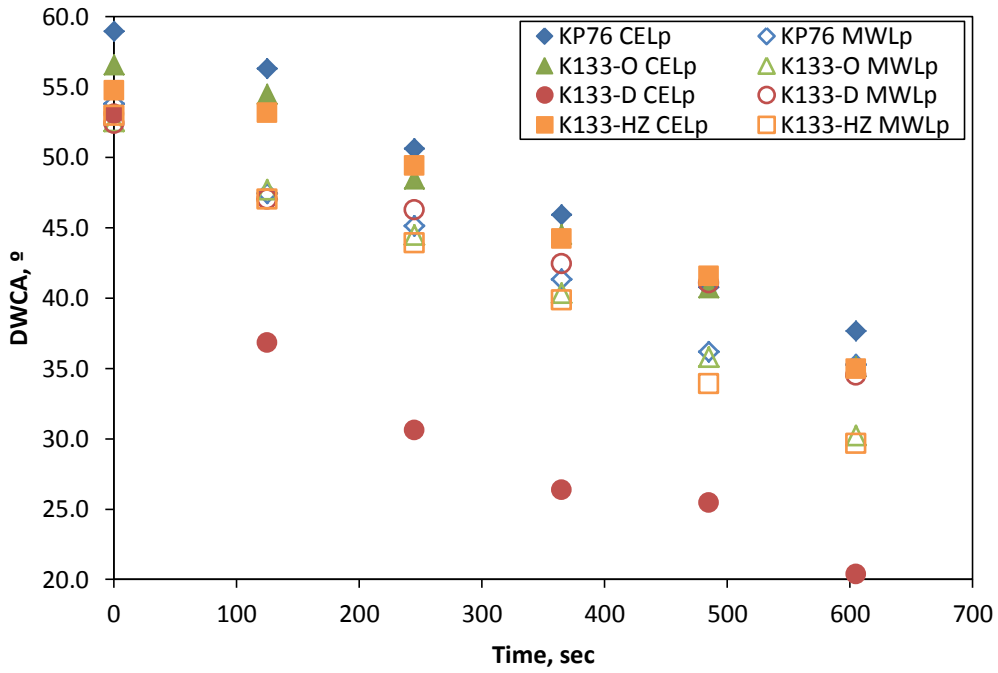

Fig. 6.9 Dynamic water contact angle (DWCA) of various CELp and MWLp

A strong correlation between initial WCA of CELp and sugar conversions was identified, Fig. 6.10. The more hydrophilic the lignin is, higher sugar conversion is achieved with respect to its corresponding pulp. One study reported that more hydrophilic lignin reduces its coordination affinity that causes nonspecific binding of cellulose (Lou et al., 2013). It indicates that lignin hydrophobicity plays an important role in enzymatic hydrolysis because hydrophobic interactions have been reported to be the most important interaction between enzymes and cellulosic fibers (Börjesson et al., 2007).

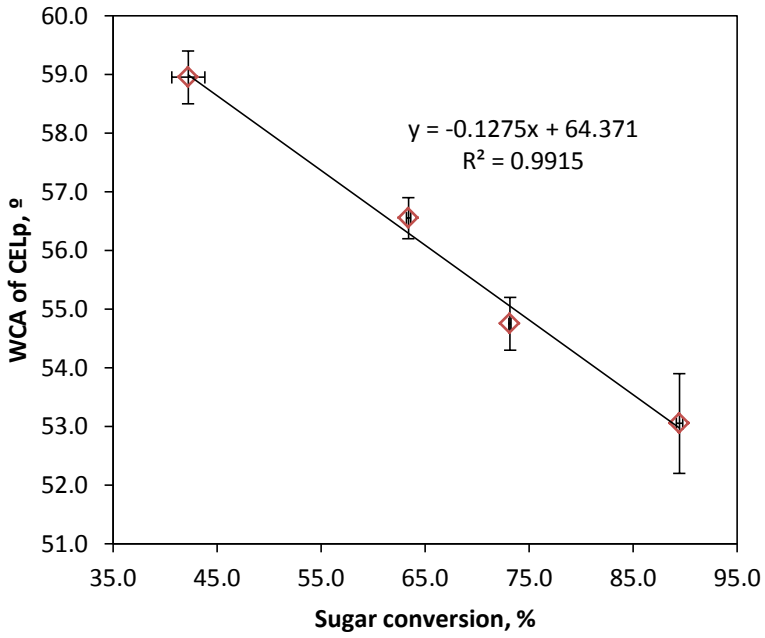

Fig. 6.10 Correlation between sugar conversion (5 FPU/g enzyme dosage) of softwood pulp with similar bulk lignin content and initial water contact angle (WCA) of the corresponding isolated CELp

Conclusions

Softwood has natural resistance to biological degradation using cellulolytic enzymes as compared to hardwoods or non-woody biomass either due to its morphological structure or

chemical compositions. As a result, most pretreatments for softwood are not capable of producing bioethanol economically due to the high amount of enzymes necessary for the conversion of carbohydrates to monomeric sugars. Softwood is a major wood resource in many parts of the world and it is difficult to envision a biofuels industry where plantations of softwoods do not play a significant role.

Softwood treated with kraft pulping, sodium chlorite, oxygen and high consistency ozone delignification show that softwood fibers at the same bulk lignin content using different treatments can show drastically different enzymatic hydrolysis efficiencies. Sodium chlorite was the most efficient pretreatment for softwood, while kraft pulping achieved the lowest enzymatic hydrolysis efficiency. In order to understand the discrepancy in enzymatic hydrolysis of softwood, the effect of lignin properties in softwood on enzymatic hydrolysis was studied. Results suggest that either accessible pore volume or water retention value (WRV) had a correlation with enzymatic hydrolysis. Lignin isolated from softwood samples with approximately equal bulk lignin contents (11.3%-13.7%) show that lignin hydrophobicity has a linear relationship with sugar conversions. Among various hydroxyl functional groups in lignin, phenolic hydroxyl groups in lignin were identified as an inhibitor to enzymatic hydrolysis. However, the total hydroxyl groups and aliphatic hydroxyl groups in softwood lignin favored sugar conversions.

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Chapter 7 Future Work

A major obstacle in the enzymatic conversion of lignocellulose is the adsorption of significant amounts of enzyme on exposed lignin surfaces. This was reported as a result of unspecific binding of cellulases by hydrophobic interactions with lignin (Eriksson et al., 2002). From the study in Chapter 6, we know that lignin hydrophobicity plays an important role in enzymatic hydrolysis of softwood and it has a strong linear correlation with sugar conversion of the softwood fiber with approximately the same bulk lignin content.

In order to study the kinetics behind enzyme adsorption to lignin, Quartz Crystal Microbalance (QCM) can be exploited to study enzyme adsorption and activity on isolated lignin coated gold electrodes. The same method of preparing spin coated lignin films used in Chapter 6 can also be applied. Since the hydrophobicity of the purified mill wood lignin (MWLp) became similar due to the hydrolysis reaction during ball milling, CELp should be used for the kinetic study.

QCM can monitor the vibration frequency of the model substrate (lignin film in this case) as a function of time. QCM experiments can reveal factors in two aspects: (a) the extent of adsorption and adsorption kinetics for the respective enzyme in contact with the model film and, (b) the affinity between enzyme and the substrate by monitoring the mass of enzyme released upon rinsing (Hu et al., 2009). Thus, an enzyme cocktail, as well as individual pure enzyme components (EG, CBH I, CBH II, BG etc.) can be studied respectively on their adsorption behavior with lignin at different hydrophobicity levels. It is also interesting to utilize SDS-PAGE analysis to monitor the enzyme inhibition by lignin. By using the isolated

lignin in Chapter 6, enzyme adsorption experiment can be conducted using enzyme cocktails with known individual enzyme component. Band intensities of each enzyme component can be compared before and after their interaction with CEL. This will provide information on variation of cellulosic enzyme activity with the presence of softwood lignin (Hu et al., 2010). These proposed experiments would provide a closure on the effect of lignin hydrophobicity on the lignin-enzyme interaction and enzymatic hydrolysis.

In addition, it was identified in this research that ash negatively impacted enzymatic hydrolysis of waste paper materials. Both acid soluble and acid insoluble ash adsorb cellulase enzyme with higher affinity than cellulose. Similar techniques on kinetic studies through QCM can also be used to examine whether the adsorption between ash compounds and enzyme is irreversible. This experiment can provide a comprehensive understanding on ash-cellulase interaction and its potential impact on single enzyme component and enzyme cocktail.

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