ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Enhanced Enzymatic Hydrolysis of Spruce by Alkaline Pretreatment at Low Temperature

Yulin Zhao,[1] Ying Wang,[1] J.Y. Zhu,[2] Art Ragauskas,[3] Yulin Deng[1]

[1]School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, Georgia, 30332; telephone: 404 894 5759; fax: 404 894 4778; e-mail: yulin.deng@chbe.gatech.edu

[2]USDA Forest Service, Forest Products Laboratory, One Gifford Pinchot Drive, Madison, Wisconsin

[3]School of Chemistry & Biochemistry, Georgia Institute of Technology, Atlanta, Georgia

Received 20 August 2007; revision received 9 October 2007; accepted 23 October 2007

Published online 19 November 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21712

ABSTRACT: Alkaline pretreatment of spruce at low temperature in both presence and absence of urea was studied. It was found that the enzymatic hydrolysis rate and efficiency can be significantly improved by the pretreatment. At low temperature, the pretreatment chemicals, either NaOH alone or NaOH–urea mixture solution, can slightly remove lignin, hemicelluloses, and cellulose in the lignocellulosic materials, disrupt the connections between hemicelluloses, cellulose, and lignin, and alter the structure of treated biomass to make cellulose more accessible to hydrolysis enzymes. Moreover, the wood fiber bundles could be broken down to small and loose lignocellulosic particles by the chemical treatment. Therefore, the enzymatic hydrolysis efficiency of untreated mechanical fibers can also be remarkably enhanced by NaOH or NaOH/urea solution treatment. The results indicated that, for spruce, up to 70% glucose yield could be obtained for the cold temperature pretreatment (−15°C) using 7% NaOH/12% urea solution, but only 20% and 24% glucose yields were obtained at temperatures of 23°C and 60°C, respectively, when other conditions remained the same. The best condition for the chemical pretreatment regarding this study was 3% NaOH/12% urea, and −15°C. Over 60% glucose conversion was achieved upon this condition.

Biotechnol. Bioeng. 2008;99: 1320–1328.

KEYWORDS: lignocellulosic materials; cellulose; biofuel; alkaline; pretreatment

Introduction

The increasing shortage of energy due to the exorbitant use of fossil oil has driven the search for alternative energy sources from renewable materials. Utilization of lower-value substrates such as lignocellulose offers a great potential for reducing the raw material cost and increasing the use of ethanol as a fuel additive (Oliva et al., 2005; Sassner et al., 2005; Stenberg et al., 1998; Varga et al., 2004).

Lignocellulosic biomass is by far the most abundant raw material from hardwood, softwood, grasses, and agricultural residues. However, unlike starch and sucrose that can be easily broken down into the carbohydrate components, pretreatment is required for the utilization of lignocellulosic materials in a bioconversion process involving enzymatic hydrolysis followed by fermentation. Overcoming recalcitrance of lignocellulosic biomass for converting carbohydrates into intermediates that can subsequently be converted into biobased fuels and biobased products is the primary technical and economic challenge for bioconversion process (Eggeman and Elander, 2005; Kaar and Holtzapple, 2000; Mosier et al., 2005; Sun and Cheng, 2002; Wilke et al., 1981; Wyman et al., 2005a,b).

Softwood materials are the most abundant feedstock available for bioconversion in many northern countries. However, the high costs for pretreatment and enzymatic hydrolysis currently deter commercialization of softwood bioconversion processes. In principal, softwood lignocellulose is considered as the most recalcitrant material for enzymatic hydrolysis mainly due to the high lignified structure and the nature of the lignin component (Pan et al., 2005).

Many pretreatment methods have been studied in literature. Among these methods, mild alkaline and dilute acid pretreatments are more practically useful due to fewer drawbacks when compared with others. However, the dilute acid pretreatment generates corrosion problems. In addition, dilute acid process produces "toxic" intermedi-

Correspondence to: Y. Deng

ates, such as furfural and aldehydes at higher temperatures, which significantly reduces the sugar yield. Furthermore, dilute acid pretreatment involves slow cellulose digestion by enzymes and nonproductive binding of enzymes to lignin. Steam explosion has been intensively studied as a promising pretreatment method for bioconversion of softwood biomass. The steam explosion is operated at high temperature and high pressure, followed by rapid reduction in pressure, to achieve fibrillation. However, some water soluble compounds released by steam explosion at high temperature and high pressure are inhibitory to microorganisms used for fermentation process (Cullis et al., 2002; Robinson et al., 2003; Yang et al., 2002). Furthermore, steam explosion technique requires costly capital investment at the equipment installation stage. Organosolv pretreatment has been viewed as another effective method for bioconversion of softwoods (Kurabi et al., 2005; Mabee et al., 2006; Pan, 2005; Pan et al., 2007). In organosolv pretreatment, lignocellulosics was treated by the mixture of water, solvent, and catalyst. The catalyst and water hydrolyze the lignin–lignin and lignin–carbohydrate bonds and the solvent creates an organophilic environment in which the lignin dissolves. However, solvent recovery and high cost of capital investment limit the further development of this method.

So far, there is no commercially feasible solution to efficient pretreatment of softwood materials for the production of biofuels and biobased products (Ahring et al., 1999; Chang et al., 2001; Reith et al., 2002). Breakthroughs in pretreatment are still needed for both scientific and economic reasons. The overall objective of this research was to develop an efficient alkaline pretreatment method for bioconversion of lignocellulosics. Spruce species was studied as substrate because it is an abundant softwood biomass in the northern hemisphere.

Sodium hydroxide and sodium hydroxide/urea at cold temperature has been studied for dissolution of cellulose (Cai and Zhang, 2005; Isogai and Atalla, 1998; Laszkiewicz, 1998; Zhang et al., 2002; Zhou et al., 2002, 2004, 2006). Our previous study reported the effect of enzymatic treatment on cotton fiber dissolution in NaOH/urea solution at cold temperature (Wang et al., 2007). The results indicated that cellulose dissolution could be significantly improved by the combination of enzyme tailoring and chemical treatment using sodium hydroxide/urea at cold temperature. However, this novel alkaline treatment technique using sodium hydroxide/urea has not been studied for application in bioconversion of lignocellulosics into biofuels and biobased products. In this article, the feasibility of using sodium hydroxide and sodium hydroxide/urea to pretreat lignocellulosic materials for bioconversion will be evaluated. The dissolution of spruce wood components, such as cellulose, hemicelluloses, and lignin using the proposed approach will be fundamentally investigated. Enzymatic hydrolysis of pretreated wood particles will also be extensively studied.

Experimental

Materials

Fresh spruce chips were donated by the Wisconsin Rapids mill of Stora Enso North America. The chips were kept frozen before use. The chips were screened to remove all particles greater than 38 mm and less than 6 mm in length. The thickness of the accepted chips ranged from 1 to 5 mm. Celluclast 1.5 L (cellulase) and Novozym 188 (β-glucosidase) were kindly provided by Novo Nordisk (Bagsværd, Denmark). Sodium hydroxide and urea were used as received from Sigma–Aldrich (St. Louis, MO).

Mechanical Size Reduction

To facilitate the penetration of chemicals used for the subsequent chemical pretreatment, spruce chips were refined using the pressurized refiner (12-inch Andritz Sprout-Bauer Pressurized Refiner, Www.Papermillsurplus.-COM, St. Louis Park, MN) at the USDA Forest Products Laboratory, Madison, WI. Spruce wood chips were first saturated in a digester before going through size reduction through mechanical refining. The wood chip loaded digester was heated (purged) with steam until the temperature of the digester reached 100°C. Then the digester was vacuumed to about 25 inches of mercury for about 10 min. The digester was then filled with hot water to about 40% solids. The mechanical refining was carried out at the following conditions: disk gap setting of 0.2 mm (0.008 inches), steam pressure 7.2184 bar (90 psig) and temperature 166.2°C. The mechanical energy consumption of the refining process was 106 Wh/kg OD wood. To simulate continuous refining process, the refiner was modified and equipped with a blow line for pulp collection. Each refining run used 5 kg OD wood chips.

Chemical Pretreatment Using NaOH or NaOH/Urea

Size-reduced wood fiber bundles were chemically pretreated using sodium hydroxide alone or sodium hydroxide combined with urea. Size-reduced wood fiber bundles were soaked in a various concentrations of sodium hydroxide solution (1%, 2%, 3%, 7%, and 12%), or soaked in some conditions of sodium hydroxide (1%, 3%, and 7%) and 12% urea solution. The weight ratio of liquid/wood solid used is 20. Fiber bundles were treated under three different temperatures, −15°C, 23°C (room temperature), and 60°C. In the case of chemical treatments at room temperature and cold temperature, 24 h duration time was used. However, 2 h treatment was used for high temperature treatment because too many carbohydrates and lignin were dissolved in the alkaline solution at high temperature for a long treatment. The chemically treated pulp was then washed with DI water to remove chemicals and neutralized to pH 7. The

neutralized samples were squeezed and conditioned for further use.

Enzymatic Hydrolysis

Enzymatic hydrolysis of the pretreated wood fiber bundles was carried out at 2% of wood component (w/v) in 50-mL sodium acetate buffer using a rotary shaker at 200 rpm. The pH and temperature were adjusted to 4.8 and 50°C, respectively. A mixture of Celluclast 1.5 L with an activity loading of approximately 20 FPU/g and Novozym 188 with an activity loading of approximately 25 CBU/g in relation to dry weight of wood fiber bundles was used for enzymatic hydrolysis. An excess of Novozym 188 was used to prevent cellobiose accumulation (Emmel et al., 2002). Reaction mixtures were pre-incubated for half hour prior to the addition of enzymes. Hydrolyzates were sampled periodically for sugar analysis. Each data point was averaged from two replicates.

Analytical Methods

Cellulase activity of Celluclast 1.5 L was determined by the filter paper method (FPU) (Wood and Bhat, 1988). Whatmann I filter paper was used as a standard substrate. One unit (FPU) of enzyme activity is defined as the amount of reducing sugars equivalent to glucose in mM/min by 1 mL of the initial enzymatic solution. Cellobiase of Novozym 188 was determined using cellobiose as substrate recommended by IUPAC Biotechnology Commission. One unit (CBU) of activity is defined as the enzyme amount, which converts 1 mol of cellubiose to 2 mol of glucose in 1 min (Wood and Bhat, 1988).

The hydrolytic reaction was followed by measuring the carbohydrates in the hydrolyzates. Sugars in the hydrolyzates were analyzed by High Performance Anion Exchange Chromatograph fitted with Pulsed Amperometric Detection (HPAEC-PAD). The system consists of a Dionex HPLC system equipped with a gradient pump, a Carbopac^{TM} PA10 column and an ED40 electrochemical detector and an auto sampler and a pneumatic controller. Klason lignin was determined according to the TAPPI standard method T-222. Acid-soluble lignin was not determined in this study. The hydrolysates from the Klason lignin measurement were collected for sugar analysis using HPLC.

Results and Discussion

The Effect of NaOH and NaOH/Urea Pretreatments on the Wood Composition and Structure

Sodium hydroxide treatment has been studied extensively to improve enzymatic hydrolysis by removing carbohydrates and lignin from lignocellulosics right after steam-exploded pretreatment (Montane et al., 1998; Pan et al., 2005). However, the traditional sodium hydroxide treatment is ineffective since it is generally performed at high temperate. This paper aimed at improving biological conversion of cellulose into glucose by using sodium hydroxide to pretreat softwood species at low temperatures because low temperatures could enhance dissolution of carbohydrates. It has been found that the addition of urea in NaOH solution could further improve the dissolution of cellulose at low temperature (Laszkiewicz, 1998). In this study, both NaOH alone and the combination of NaOH and urea were used as pretreatment solutions, and the temperature effect on the pretreatment system was investigated.

Slightly refining with a pressurized refiner was used as the method for woodchip size reduction. Figure 1 shows that fiber bundles with the width of a couple of hundreds of microns and the length of several millimeters were obtained by the refining process. The chemical compositions of wood fiber bundles, before and after chemical treatment were analyzed as listed in Table I. Table I also listed the percentage of weight loss of each chemical component (in the parentheses) through different pretreatment processes. The weight loss of other chemical components can be seen from the difference between the sum of the four components analyzed and the measured sample mass (the last column). For the untreated sample, the difference is mainly attributed to wood extractive that was removed in the alkaline pretreatment processes.

Untreated spruce mainly consists of lignin, cellulose, mannan, and xylan. Only small amounts of arabinan and galactan were detected. For wood fiber bundles, the treatment removed ca. 19% lignin, 40% xylan, and small amount of mannan, which is not strongly dependant on the treatment method (Table I).

Figure 2 shows the fiber physical structure changes after sodium hydroxide/urea treatment at cold temperature. The SEM figures clearly show that loose structure of spruce fiber bundles was created by this pretreatment compared to the untreated fiber bundles. Moreover, the fiber bundles were slightly broken down to small pieces by the chemical treatment. It should be noted that the loose structure of wood fiber bundles and smaller particle size generated by pretreatment should allow for hydrolytic enzymes to penetrate and hydrolyze the wood fibers easily, thus the enzymatic hydrolysis efficiency of the fiber bundles could be enhanced.

The Effects of NaOH or NaOH/Urea Pretreatments on Enzymatic Hydrolysis of Cellulose

Spruce fiber bundles treated by NaOH or NaOH/urea at three selected temperatures (−15°C, 23°C, 60°C) were subjected to enzymatic hydrolysis. The results shown in Figure 3 reveals that treatment temperature greatly affected enzymatic hydrolysis, and the cold temperature significantly increased the enzymatic hydrolysis efficiency. Over 60% theoretical glucose yield could be achieved from enzymatic

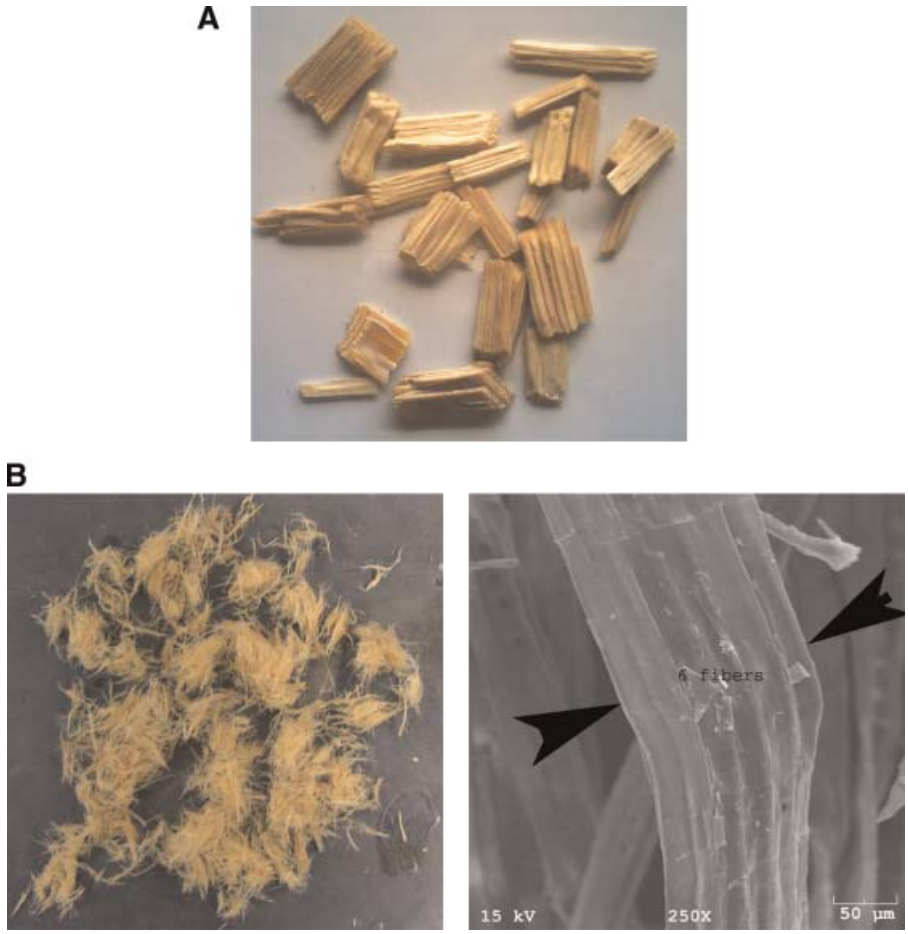

Figure 1. (A) Wood chips and (B) wood fiber bundles after refining. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

hydrolysis of cold NaOH treated fiber bundles for 7 days at the enzymes loading amounts of 20 FPU/25 CBU. The results also indicate that combining urea with sodium hydroxide could further increase the enzymatic hydrolysis efficiency under all the temperatures studied. When pretreatment was conducted under freezing conditions (−15°C) with sodium hydroxide and urea, almost 70% theoretical glucose yield was obtained.

The effect of the dosage of sodium hydroxide used in pretreatment at cold temperature (−15°C) on the enzymatic hydrolysis efficiency was studied. Figure 4 shows two distinct steps of bioconversion of cellulose-to-glucose by enzymatic hydrolysis. An initial quick enzymatic hydrolysis step occurred in the first 2 days and then was followed by a slow enzymatic hydrolysis reaction. Most glucose was produced in the first 2 days. Glucose yield increase was not very significant from 2 to 7 days. It was observed that the hydrolysis rate dropped quickly in the first 2 days, and the hydrolysis rate was only slightly changed for the rest of days as shown in Figure 5.

Table I. Weight loss of wood components after chemical treatments.

Chemical treatments	Klason lignin (g and % reduction)	Glucan (g and % reduction)	Xylan (g and % reduction)	Mannan (g and % reduction)	Sum (g)	Total sample (g)
Untreated sample	3.060	4.976	0.540	1.035	9.611	10
24 h at −15°C						
7% NaOH	2.480 (19.0%)	4.623 (7.1%)	0.327 (39.4%)	0.998 (3.5%)	8.428	8.55
7% NaOH + 12% urea	2.457 (19.7%)	4.683 (5.9%)	0.318 (41.2%)	1.007 (2.7%)	8.465	8.50
24 h at 23°C						
7% NaOH	2.488 (18.7%)	4.552 (8.5%)	0.323 (40.2%)	1.019 (1.5%)	8.382	8.52
2 h at 60°C						
7% NaOH	2.462 (19.5%)	4.550 (8.6%)	0.316 (41.4%)	1.017 (1.8%)	8.345	8.55

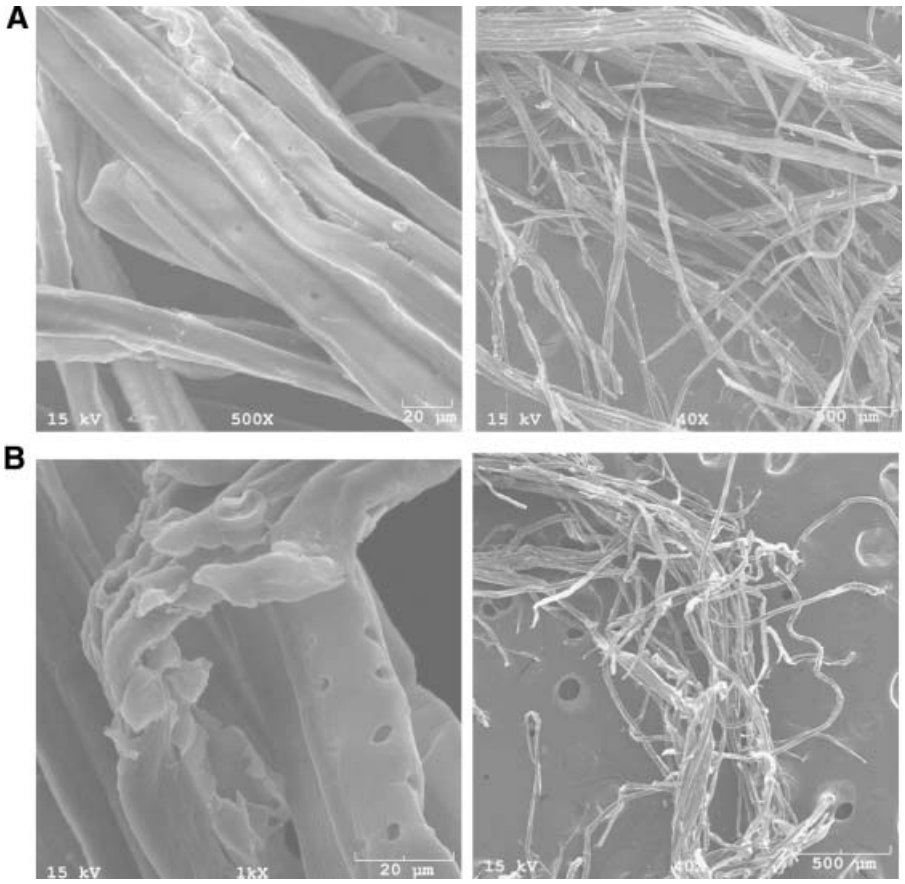

Figure 2. SEM of wood fiber bundles before (A) or after NaOH/urea treatment at cold temperature (B).

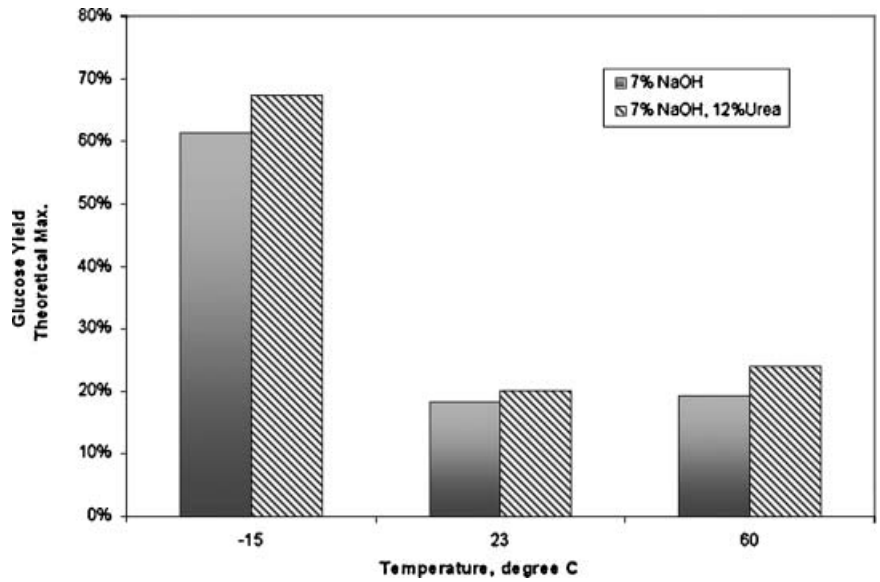

Figure 3. Temperature effect of chemical treatment on bioconversion of cellulose-to-glucose (20 PFU/25 CBU).

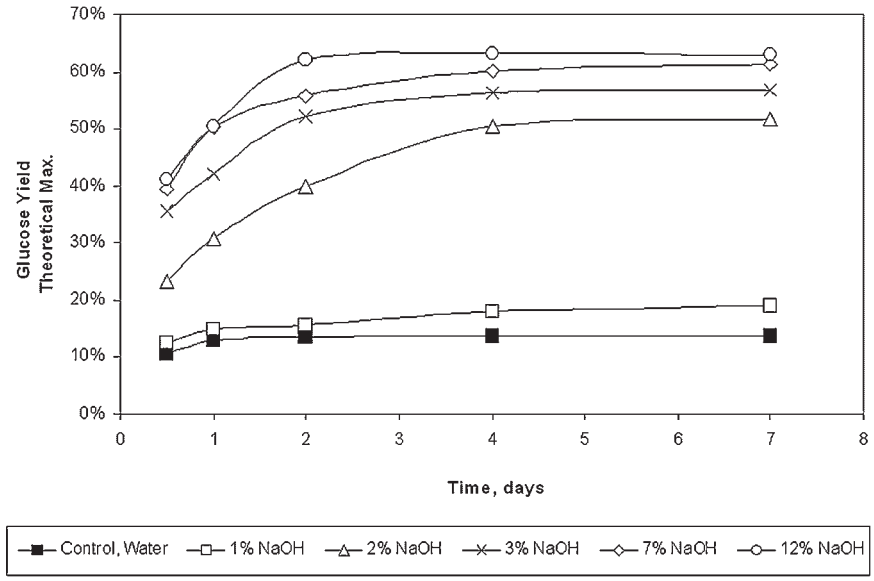

Figure 4. Effect of loading amount of sodium hydroxide on bioconversion of cellulose-to-glucose (cold temperature, 20 PFU/25 CBU).

Figures 4 and 5 also indicated that increased sodium hydroxide dosage enhanced the glucose yield. Two percentage sodium hydroxide is a critical dosage because enzymatic hydrolysis rate and efficiency was dramatically increased for this loading amount compared to 1% dosage. Continuously increasing sodium hydroxide dosage further improved the bioconversion of cellulose-to-glucose. However, no significant improvement was found when the dosage of sodium hydroxide was increased from 7% to 12%. Even though initial enzymatic hydrolysis rate increased remarkably by the increased sodium hydroxide dosage, the hydrolysis rate dropped quickly after the first 2 days followed by a steady decrease. The enzymatic hydrolysis rate decreased to a constant rate after 7 days for all sodium hydroxide loadings greater than 2% (Fig. 5).

The spruce fiber bundles were treated by the combination of urea with sodium hydroxide at different loading amounts at -15°C and then were hydrolyzed by the enzymes. The results reveal that the combination of urea with sodium hydroxide produced a similar enzymatic hydrolysis trend

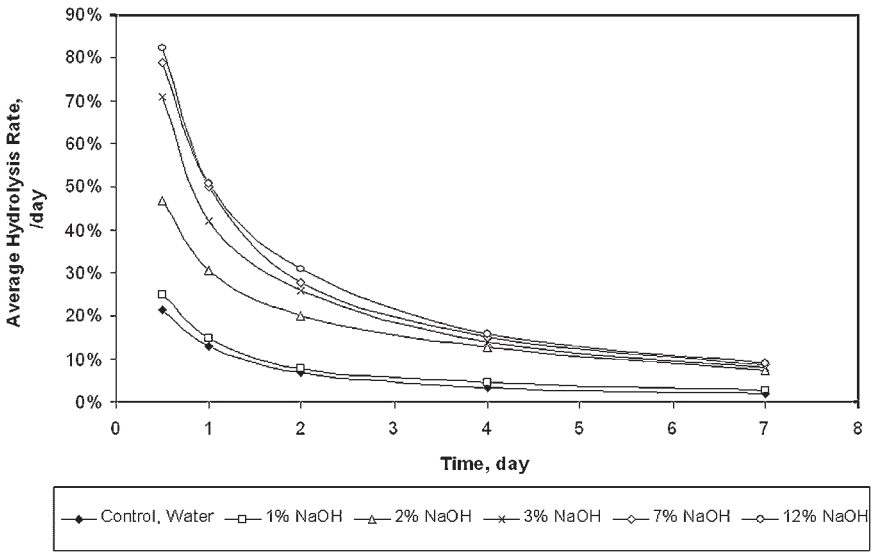

Figure 5. Effect of loading amount of sodium hydroxide on average hydrolysis rate (glucose yield/incubation time, %/day).

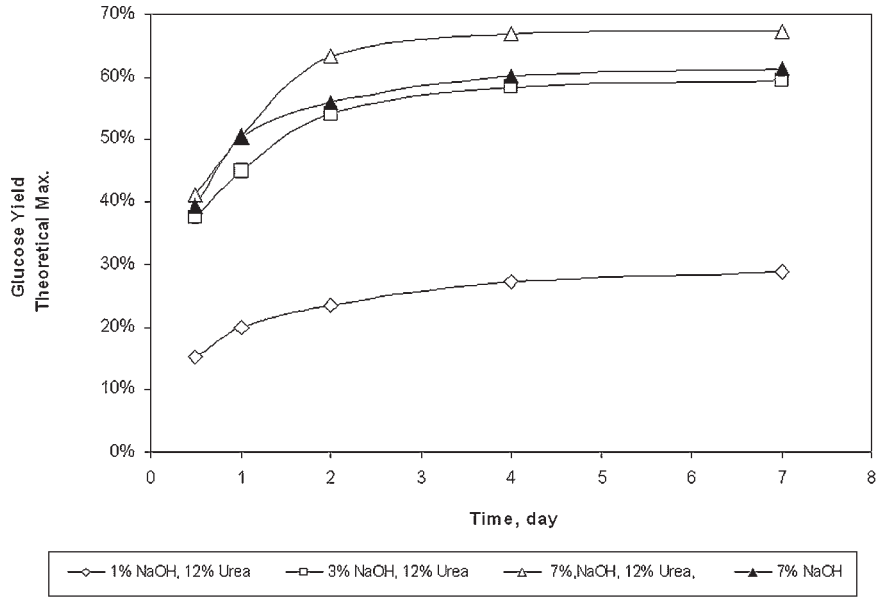

Figure 6. Effect of loading amount of sodium hydroxide combined with urea on bioconversion of cellulose-to-glucose (cold temperature, 20 PFU/25 CBU).

to that obtained using sodium hydroxide alone (Fig. 6). Two distinct hydrolysis steps were observed: a quick enzymatic hydrolysis step followed by a slow enzymatic hydrolysis reaction. However, the addition of urea extended the first step to 3 days before entering the second slow reaction step. As a result, the bioconversion of cellulose to glucose was increased about 8% when urea was added in pretreatment. The loading amount of sodium hydroxide significantly affected glucose yield by the enzymatic hydrolysis. Similar to the sodium hydroxide alone approach, there is a critical dosage of sodium hydroxide for this combination method. Three percentage loading amount of sodium hydroxide with 12% loading amount of urea significantly improved the enzymatic hydrolysis of pretreated spruce fiber bundles compared with the 1% sodium hydroxide with 12% urea dosage.

The Effects of NaOH or NaOH/Urea Pretreatments on Enzymatic Hydrolysis of Hemicelluloses

Enzymatic hydrolysis of hemicelluloses of wood bundles after chemical treatments was also investigated. As mentioned before, mannan, and xylan are the main components of hemicelluloses in spruce. Therefore, the fates of mannan and xylan during enzymatic hydrolysis were compared between the wood bundles treated at different temperature using NaOH and NaOH/urea.

The results shown in Figures 7 and 8 indicate that enzymatic hydrolysis of xylan and mannan were significantly affected by treatment temperatures, and the cold temperature greatly increased enzymatic hydrolysis efficiency. Over 70% theoretical xylose yield and about 55% theoretical mannose could be achieved from enzymatic hydrolysis of cold NaOH treated fiber bundles for 7 days at the enzymes loading amounts of 20 FPU/25 CBU. The results also show that combining urea with sodium hydroxide could further increase the enzymatic hydrolysis efficiency under all the temperatures studied. When pretreatment was conducted under freezing conditions (−15°C) with sodium hydroxide and urea, about 80% theoretical xylose yield and 60% mannose yield were obtained.

It should be noted that the amount of NaOH and urea used in this study may be too high for actual industrial application. To solve these problems, several approaches, including NaOH recovery, partially substitution of NaOH

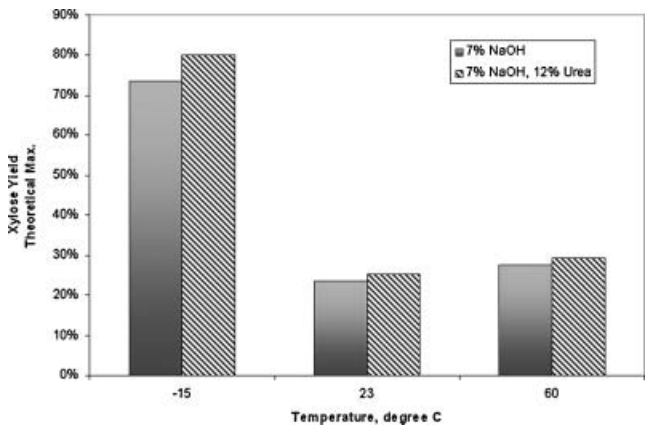

Figure 7. Temperature effect of chemical treatment on bioconversion of xylan-to-xylose (20 PFU/25 CBU).

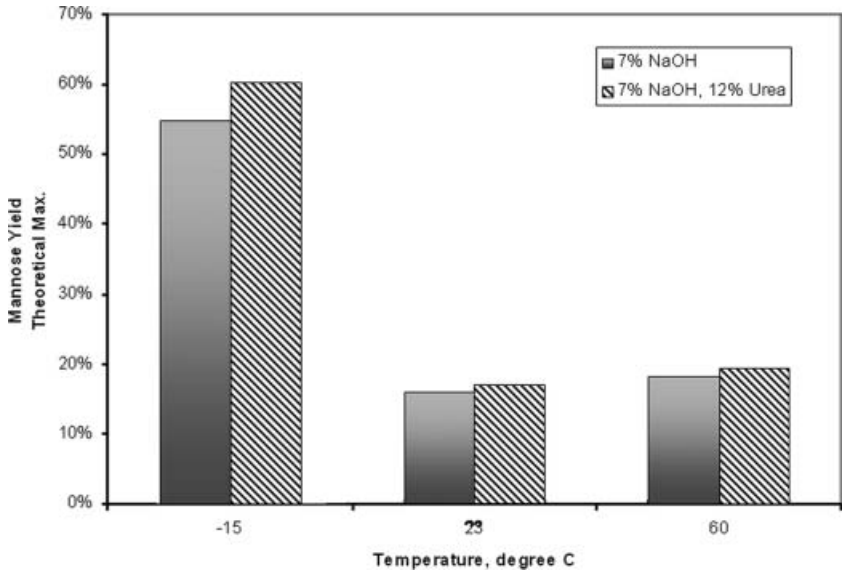

Figure 8. Temperature effect of chemical treatment on bioconversion of mannan-to-mannose (20 PFU/25 CBU).

using black liquor from Kraft pulping, and using CaO or $Ca(OH)_2$ etc. may be used. All these approaches are being tested in our laboratory now. It was also realized that it is difficult to low the operation temperature in a large scale industrial operation. However, there may be also some promising approaches for solving this problem. For example, some of our experiments were carried out in Madison, Wisconsin. We simply left the samples outside the building during winter for overnight. Experimental results indicated that the winter temperature in northern region is low enough to cool the system to required temperature. Therefore, the technology may be used in north area during winter season without spending extra energy for cooling the pretreatment system.

Conclusions

The overall purpose of this paper is to explore a new pretreatment method to improve the enzymatic hydrolysis of spruce, and increases the theoretical glucose conversion yield. Sodium hydroxide or sodium hydroxide combined with urea at cold temperature to dissolve cellulose from cotton and wood pulps has been studied. To our best knowledge, no similar approach has been reported in literature.

The structure and the components of spruce before and after chemical treatment were determined. Success of the pretreatment for the softwood biomass appears to result predominantly from the looser structure and smaller wood bundles to allow penetration of cellulolytic enzymes. Another possible reason might be the cleavage of lignin–carbohydrate bonds and increased pore volume.

Sodium hydroxide treatment at frozen temperature could enhance the susceptibility of substrate to enzymatic hydrolysis. Enzymatic hydrolysis efficiency and rate could be significantly increased by this approach. Bioconversion efficiency of cellulose-to-glucose was improved by increasing the loading amount of sodium hydroxide. Increase NaOH concentration resulted in higher hydrolysis degree. However, no significant improvement was found if the dosage of sodium hydroxide was further increased beyond 7% in solution. The combination of urea with sodium hydroxide could further enhance enzymatic hydrolysis of softwood biomass. Almost 70% theoretical glucose yield could be achieved by using 7% sodium hydroxide and 12% urea treatment at cold temperature.

Enzymatic hydrolysis of hemicelluloses of spruce bundles after chemical treatments was also investigated. It was also found that the enzymatic hydrolysis of xylan and mannan was significantly affected by treatment temperatures, and the cold temperature greatly increased enzymatic hydrolysis efficiency. When pretreatment was conducted under freezing conditions ($-15^{\circ}C$) with sodium hydroxide and urea, about 80% theoretical xylose yield and 60% mannose yield were obtained.

Theoretically speaking, softwood lignocellulose is considered as the most recalcitrant material for enzymatic hydrolysis mainly because of the high lignified structure and the nature of the lignin component. Also, its higher content of guaicyl units promotes condensation during pretreatment. Therefore, the developed pretreatment method in this study can be used for many kinds of lignocellulosic materials such as wood chips, sawdust, corn stover, rice straw, paper sludge, waste papers, and switchgrass for the productions of biofuels and biobased products.

References

Ahring BK, Licht D, Schmidt AS, Sommer P, Thomsen AB. 1999. Production of ethanol from wet oxidized wheat straw. Bioresource Technol 68:3–9.

Cai J, Zhang L. 2005. Rapid dissolution of cellulose in LiOH/Urea and NaOH/Urea aqueous solutions. Macromol Biosci 5(6):539–548.

Chang VS, Kaar WE, Bun B, Holtzapple MT. 2001. Simultaneous saccharification and fermentation of lime-treated biomass. Biotechnol Lett 23(16):1327–1333.

Cullis IF, Saddler JN, Mansfield SD. 2002. Effect of initial moisture content and chip size on the bioconversion efficiency of softwood lignocellulosics. Biotechnol Bioeng 85(4):413–421.

Eggeman T, Elander RT. 2005. Process and economic analysis of pretreatment technologies. Bioresource Technol 96:2019–2025.

Emmel A, Mathias AL, Wypych F, Ramos LP. 2002. Fractionation of Eucalyptus Grandis chips by dilute acid-catalysed steam explosion. Bioresource Technol 86:105–115.

Isogai A, Atalla RH. 1998. Dissolution of cellulose in aqueous NaOH solutions. Cellulose 5(4):309–319.

Kaar WE, Holtzapple MT. 2000. Using lime pretreatment to facilitate the enzymatic hydrolysis of corn stover. Biomass Bioeng 18:189–199.

Kurabi A, Berlin A, Gilkes N, Kilburn D, Bura R, Robinson J, Markov A, Skomarovsky A, Gusakov A, Okunev O, Sinitsyn A, Gregg D, Xie D, Saddler J. 2005. Enzymatic hydrolysis of steam-exploded and ethanol organosolv-pretreated Douglas-fir by novel and commercial fungal cellulases. Appl Biochem Biotechnol Enzym Eng Biotechnol 121–124:219–230.

Laszkiewicz B. 1998. Solubility of bacterial cellulose and its structural properties. J App Polym Sci 67(11):1871–1876.

Mabee WE, Gregg DJ, Arato C, Berlin A, Bura R, Gilkes N, Mirochnik O, Pan X, Pye EK, Saddler JN. 2006. Updates on softwood-to-ethanol process development. Appl Biochem Biotech 129(1–3):55–70.

Montane D, Farriol X, Salvado J, Jollez P, Chornet E. 1998. Fractionation of wheat straw by steam-explosion pretreatment and alkali delignification: Cellulose pulp and byproducts from hemicellulose and lignin. J Wood Chem Technol 18(2):171–191.

Mosier NS, Wyman CE, Dale B, Elander R, Lee YY, Holtzapple M, Ladish M. 2005. Features of promising technologies for pretreatment lignocellulosic biomass. Bioresource Technol 96:673–686.

Oliva JM, Manzanares P, Ballesteros I, Negro MJ, Gonzalez A, Ballesteros M. 2005. Aplication of Fenton's reaction to steam explosion prehydrolysates from poplar biomass. J Appl Biochem Micro Part A Enzyme Eng Biotech 121–124:887–899.

Pan X. 2005. Ethanol from organosolv softwood biorefining. Ind Bioproces 27(4):7–8.

Pan X, Xie D, Gilkes N, Gregg JD, Saddler NJ. 2005. Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. Appl Biochem Biotech 121–124:1069–1079.

Pan X, Xie D, Yu RW, Lam D, Saddler JN. 2007. Pretreatment of lodgepole pine killed by mountain pine beetle using the ethanol organosolv process: Fractionation and process optimization. Ind Eng Chem Res 46(8):2609–2617.

Reith JH, den Uil H, van Veen H, de Laat WTAM, Niessen JJ, de Jong E, Elbersen HW, Weusthuis R, van Dijken JP, Raamsdonk L. 2002. Co-production of bio-ethanol, electricity and heat from biomass residues, 12th European Conference and Technology Exhibition on Biomass for Energy, Industry and Climate Protection. The Netherlands: Amsterdam.

Robinson J, Keating JD, Mansfield SD, Saddler JN. 2003. The fermentability of concentrated softwood-derived hemicellulose fractions with and without supplemental cellulose hydrolysates. Enzyme Microb Tech 33:757–765.

Sassner P, Galbe M, Zacchi G. 2005. Steam pretreatment of salix with and without SO_2 impregnation for production of bioethanol. Appl Biochem Biotechnol Enzym Eng Biotechnol 121–124:1101–1117.

Stenberg K, Tengborg C, Galbe M, Zacchi G. 1998. Optimization of steam pretreatment of SO_2-impregnated mixed softwoods for ethanol production. J Chem Tech Biotech 71(4):299–308.

Sun Y, Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresource Technol 83(1):1–11.

Varga E, Reczey K, Zacchi G. 2004. Optimization of steam pretreatment of corn stover to enhance enzymatic digestibility. Appl Biochem Biotechnol Enzym Eng Biotechnol 113–116:509–523.

Wang Y, Zhao Y, Deng Y. 2007. Effect of enzymatic treatment on cotton fiber dissolution in NaOH/urea solution at cold temperature. Carbohydr Polym, available online 17 August 2007.

Wilke CR, Yang RD, Sciamanna AF, Freitas RP. 1981. Raw materials evaluation and process development studies for conversion of biomass to sugars and ethanol. Biotech Bioeng 23:63–83.

Wood TM, Bhat KM. 1988. Methods for measuring cellulase activities. Methods Enzymol Biomass Part A Cellulose Hemicellulose 160:87–112.

Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. 2005a. Coordinated development of leading biomass pretreatment technologies. Bioresource Technol 96:1959–1966.

Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. 2005b. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresource Technol 96:2026–2032.

Yang B, Boussaid A, Mansfield DS, Gregg JD, Saddler JN. 2002. Fast and efficient alkaline peroxide treatment to enhance the enzymatic digestibility of steam exploded softwood substrates. Biotech Bioeng 77(6):678–684.

Zhang L, Ruan D, Gao SJ. 2002. Dissolution and regeneration of cellulose in NaOH/thiourea aqueous solution. J Polym Sci B Polym Phys 40(14):1521–1529.

Zhou JP, Zhang L, Cai J, Shu H. 2002. Cellulose microporous membranes prepared from NaOH/Urea aqueous solution. J Membr Sci 210(1):77–90.

Zhou JP, Zhang L, Cai J. 2004. Behavior of cellulose in NaOH/urea aqueous solution characterized by light scattering and viscometry. J Polym Sci B Polym Phys 42(2):347–353.

Zhou JP, Qin Y, Liu SL, Zhang L. 2006. Homogenous synthesis of hydroxyethylcellulose in NaOH/Urea aqueous solution. Macromol Biosci 6(1):84–89.