Enzymatic Saccharification of Eucalyptus Chips with a Pretreatment Process Using NH_4Cl

Dan Huo,[a,b,d,e,g]* Dongsheng Wang,[a] Qiulin Yang,[a,c,f,]* Chuanling Si,[a] Qiujuan Liu,[a] Bin Li,[g] and Fengshan Zhang [f]

NH_4Cl was used to optimize the pretreatment conditions for biomass pretreatment to improve enzymatic saccharification and hemicellulose degradation of eucalyptus chips. After pretreatment, the solid substrate (SS) and pretreatment liquor (PL) were characterized, and the SS was enzymatically hydrolyzed to detect the conversion yield of cellulose (CYC). For the pretreatment using NH_4Cl, the removal rate of hemicellulose reached up to 100% in some cases, but a great proportion of cellulose remained in the SS. The optimized conditions for pretreatment using NH_4Cl were 0.3 M NH_4Cl at 200 °C for 25 min. A comprehensive evaluation found that the most suitable severity parameter for pretreatment and enzymatic saccharification was 4.5, although a higher severity parameter could increase the CYC. XRD and FTIR analysis showed that the pretreatment had little influence on the cellulose crystalline region, and the lignin was well-retained in the pretreatment process.

Keywords: Pretreatment; NH_4Cl; Hemicellulose; Enzymatic saccharification

*Contact information: a: Tianjin Key Laboratory of Pulp & Paper, Tianjin University of Science & Technology, Tianjin 300457, China; b: Key Laboratory of Renewable Energy, Chinese Academy of Sciences, Guangzhou 510640, China; c: Jiangsu Province Biomass Energy and Materials Laboratory, Institute of Chemical Industry of Forest Products, CAF, Nanjing 210042, China; d: State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, China; e. Jiangsu Key Lab of Biomass-based Green Fuels and Chemicals, Nanjing Forestry University, Nanjing, 210042, China; f: Shandong Huatai Paper Co., Ltd., Dongying 275335, China; g: CAS Key Laboratory of Bio-based Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China; *Corresponding author: danhuo@mail.tust.edu.cn; yangqiulin@tust.edu.cn*

INTRODUCTION

As one of the main components of cell wall, cellulose is the most abundant polysaccharide in nature, and its mass accounts for approximately 1/3 to 1/2 of plant dry weight (Caffall and Mohnen 2009). Every year, approximately 4 billion tons of cellulose are synthesized by plants, making it an inexhaustible and renewable resource. Thus, it is of great importance for solving problems such as food shortages, environmental pollution, the energy crisis, and feed resource shortages that natural cellulose can be separated into simple sugars for converting into bioethanol, gaseous fuel, *etc.* (Anwar *et al*. 2014). Because of the intramolecular and intermolecular hydrogen bonds, cellulose is very difficult to dissolve using water as well as most organic solvents (Nishiyama *et al.* 2002). The purpose of biomass pretreatment is to increase the biotransformation rate with the help of physical methods, chemical methods, physical-chemical methods, biological methods, *etc.* Normally, biomass pretreatments could modify the lignin structure, change the condensation degree of the lignin, decrease the cellulose's degree of polymerization,

increase the degree of porosity and looseness of cellulose, and result in increasing enzyme accessibility to cellulose, *etc.* (Li, *et al.* 2007; Ibrahim *et al.* 2010; Rollin *et al.* 2011; Zhang *et al.* 2016a)

The chloride salt NH_4Cl dissolves easily in water to form a weakly acidic solution, and the acidity can be increased by heating. Kang *et al.* (2013a) pretreated *Miscanthus* straw using NH_4Cl to investigate xylan hydrolysis. The results show that the xylan in the raw material can be completely hydrolyzed when 2% NH_4Cl is used at 185 °C for 15 min (Kang *et al.* 2013a,b), which demonstrates that NH_4Cl has strong degradation effects on hemicellulose and can be used as a pretreatment for biomass conversion. Moreover, the PL, being rich in carbohydrates and NH_4Cl, is an excellent liquid fertilizer and has little influence on the environment.

In this study, milled eucalyptus chips were pretreated using NH_4Cl to explore the degradation of cellulose and hemicellulose, and the obtained SS was hydrolyzed using enzymes to calculate the CYC.

EXPERIMENTAL

Materials

Eucalyptus chips provided by Jingui Paper Co. Ltd. (Guangxi, China) were milled and screened to obtain a fraction of 40 mesh to 60 mesh. Cellulase (Celluclast 1.5 L, 50.97 FPU/mL) and β-glucosidase (Novozyme 188, 1290.69 CBU/mL) were obtained from Novozymes (Beijing, China). All other reagents used were of analytical grade and purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

Pretreatment with NH_4Cl

The pretreatment process was performed in custom-built tubular 316-L stainless steel reactors 45 mL in capacity. The eucalyptus powder was placed in the reaction kettle with a solid-to-liquid ratio (NH_4Cl solution) of 1:6. NH_4Cl concentration, pretreatment holding time, and pretreatment temperature were controlled in the ranges of 0.05 M to 0.5 M, 5 min to 60 min, and 140 °C to 220 °C, respectively. After pretreatment, the reactor was instantly placed in fluid cool water to terminate the reaction. The solid residue from the reactor was filtered by vacuum filtration to obtain the PL and the SS.

Characteristics of the Raw Material and the SS

The components of the raw material and the SS were analyzed according to the NREL LAP method (Sluiter *et al.* 2008). The components of the raw material were as follows: 47.86% glucan, 15.50% xylan, 1.03% arabinan, and 29.15% lignin. The SS yield was designated as the mass ratio of the SS to that of the raw material.

Enzymatic Hydrolysis

The SS was diluted with a buffer solution (0.1 M sodium acetate and 0.1 M acetic acid, pH 4.8) to keep the concentration at 2.5% (all based on the oven dried SS, o.d.). Then, 30 FPU/g of cellulase, 37.5 CBU/g of β-glucosidase, and three drops of ethyl acetate were added to the diluted substrate. The hydrolysis process was conducted in a cultivation shaker for 48 h at 50 °C, with a rotational speed of 150 rpm. After hydrolysis, the enzymatic slurry was heated for 5 min at 90 °C in a water bath to deactivate the enzymes.

Detection of Monosaccharides and Sugar Degradation Compounds

The xylose, glucose, organic acid, and sugar degradation compounds furfural and 5-hydroxymethyl furfural (5-HMF) in the PL were detected directly by HPLC. To detect monosaccharides in the enzymatic hydrolysate, it was centrifuged at 10,000 rpm for 10 min and filtered with a membrane filter (0.22 μm) prior to HPLC detection. The HPLC (Agilent 1260) (Santa Clara, CA, USA) equipped with a Bio-Rad Aminex HPX-87H (300 × 7.8 mm) (Hercules, USA) column was used, and detecting conditions were as follows: column temperature 55 °C, differential detector, mobile phase H_2SO_4 (0.05 M) with a flow rate of 0.6 mL/min, and a sampling volume of 10 μL. The CYC was calculated as follows:

$$CYC = 0.9 * (\text{glucose in enzymatic hydrolyzate}) / (\text{cellulose in the SS}) \quad (1)$$

FTIR and XRD Detection

FTIR measurements were performed in a Nicolet Magna-IR 550 Fourier transform spectrometer (Thermo Fisher, Waltham, MA), using the KBr method. The spectra were recorded over a frequency range of 4000 cm^{-1} to 600 cm^{-1} at a resolution of 2 cm^{-1} in transmission mode. For the crystallinity analysis, the ground sample (80 to 120 mesh) was measured using a D8-FOCUS horizontal X-ray diffractometer (Bruker, Billerica, MA, USA). The degree of crystallinity (CrI) was calculated as follows:

$$CrI = (I_{002} - I_{\underline{am}}) \times (100/I_{002}) \quad (2)$$

where I_{002} is the maximum intensity of the (002) lattice diffraction and I_{am} is the intensity diffraction at $2\theta = 18$ ° (Segal *et al.* 1959).

RESULTS AND DISCUSSION

Effect of Pretreatment Conditions

Pretreatment time

As shown in Table 1, when the pretreatment time was extended, the SS yield clearly declined, and its downtrend changed slowly when the pretreatment time was greater than 30 min, which indicates that most of the degradation reaction was completed in a short time. For the PL, the maximum contents of the xylose and glucose (12.38 mg/mL and 5.65 mg/mL, respectively) emerged when pretreated for 30 min. However, when the pretreatment time was prolonged further, their contents decreased remarkably. Saeman (1945) found that the degradation of hemicellulose is accomplished according to a specific pathway: hemicellulose → oligosaccharides → monosaccharides → degradation byproducts. The sustained increase in the furfural and 5-HMF contents confirmed that some xylose and glucose was dehydrated in the pretreatment process. Especially for glucose, the 5-HMF content was as high as 15.29 mg/mL when pretreated for 60 min. This demonstrated that prolonging the pretreatment time was not conducive for retaining the cellulose in the SS. Correspondingly, the converted glucose mass also decreased. The production of formic acid and acetic acid were mostly related to the hemicellulose. Their contents always increased during the pretreatment, which also explained the increasing hemicellulose degradation in the pretreatment. Based on the above analysis, the optimized pretreatment time was approximately 25 min, where the CYC reached 92.6%. In the meantime, the cellulose loss was at a relatively low level.

Table 1. Effects of Pretreatment Time on SS and PL[a]

Time (min)	SS yield (%)	PL (mg/mL)						CYC (%)
		Xy	Glu	Fa	Aa	Ff	5-HMF	
5	92.2	0.85	1.22	0.29	0.56	0.01	0.27	19.9
10	81.9	4.81	1.82	-	-	-	-	30.4
15	78.3	5.90	2.29	1.42	2.05	0.27	0.53	54.8
20	75.1	10.17	3.53	-	-	-	-	82.1
25	72.2	10.87	5.59	2.02	3.58	1.30	3.72	92.7
30	70.4	12.38	5.65	-	-	-	-	85.6
40	68.8	7.01	5.21	3.91	5.83	2.62	10.16	80.1
50	67.6	4.64	4.61	-	-	-	-	-
60	66.6	2.85	4.09	4.60	6.08	3.32	15.29	-

a: Conditions: NH_4Cl 0.3 M, solid-to-liquid ratio 1:6, and temperature 190 ºC
b: Xy – xylose; Glu – glucose; Fa – formic acid; Aa – acetic acid; Ff – furfural

Pretreatment temperature

As shown in Figs. 1 and 2, when the pretreatment temperature was increased from 140 to 220 °C, the SS yield decreased by 22.4%. Most of the degradation reactions were accomplished at a relatively lower pretreatment temperature, as the SS yield changed little when the pretreatment temperature was above 210 °C. Figure 1 shows that the effects of the pretreatment temperature on hemicellulose degradation were more severe than those on cellulose. As the pretreatment temperature increased towards 220 °C, nearly all of the hemicellulose was destroyed. At that temperature, the cellulose loss was as high as 20.7%. An obvious inflection point emerged where the cellulose loss accelerated when the pretreatment temperature was greater than 190 °C, which indicates that maintaining a lower temperature is beneficial for reserving the cellulose.

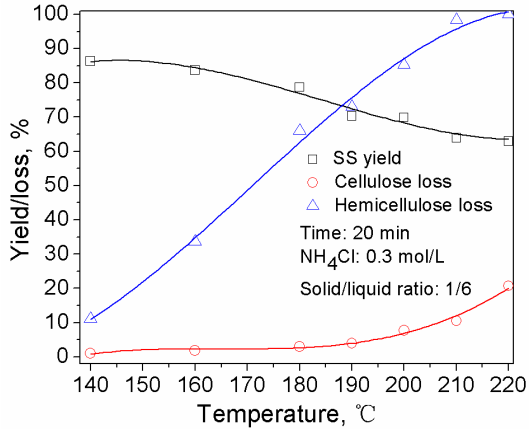

Fig. 1. Pretreatment temperature influence on the SS yield and hemicellulose/cellulose loss

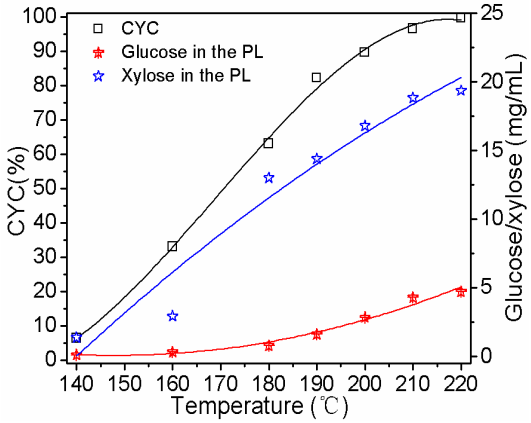

Fig. 2. Pretreatment temperature influence on the PL and the CYC

Figure 2 shows that the maximum contents of xylose and glucose in the PL occurred at 220 °C. Correspondingly, the CYC was as high as 99.5%. Higher pretreatment temperatures not only resulted in a great amount of cellulose degradation, but also destroyed the crystal structure of cellulose; thus, the CYC was at a very high level. Unlike the glucose and xylose in the PL, when the pretreatment temperature was greater than 200 °C, the increasing trend of the CYC slowed, as the CYC nearly reached the maximum value and was difficult to increase. This demonstrated that higher

pretreatment temperature was not beneficial to the CYC, as it not only caused massive hemicellulose degradation, but also resulted in rapid cellulose degradation. Hence, a pretreatment temperature of approximately 200 °C was suitable, and the CYC at this temperature was approximately 90%.

NH_4Cl concentration

The influence of NH_4Cl concentration is shown in Figs. 3 and 4. The NH_4Cl concentration had smaller impacts on SS yield and cellulose loss than it did on hemicellulose loss. When the NH_4Cl concentration of the pretreatment system increased from 0.1 M to 0.5 M, the SS yield, cellulose loss, and hemicellulose loss increased by 2.5%, 12.3%, and 59.0%, respectively. More than 86% of the hemicellulose in the raw material was degraded when the NH_4Cl concentration approached 0.5 M, which indicated that NH_4Cl concentration remarkably affected hemicellulose degradation.

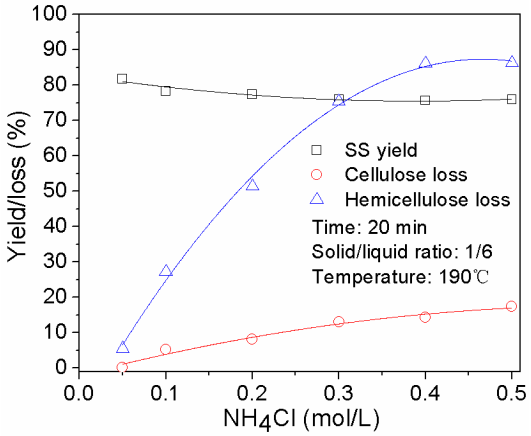

Fig. 3. NH_4Cl influence on the SS yield and hemicellulose/cellulose loss

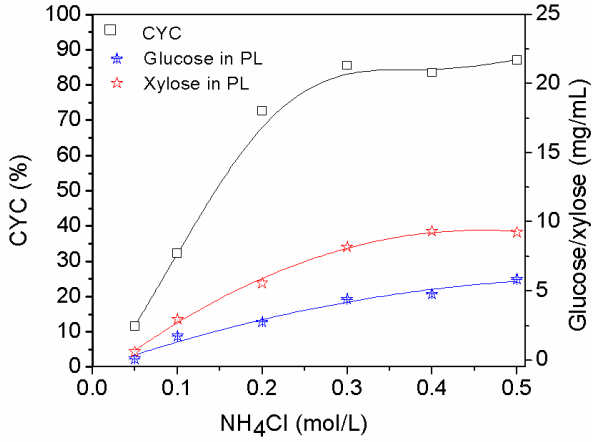

Fig. 4. NH_4Cl influence on the PL and the CYC

As shown in Fig. 4, similar to the trends for pretreatment time and pretreatment temperature, the xylose content in the PL was greater than that of the glucose. When NH_4Cl concentration was greater than 0.4 M, the xylose contents changed slightly, while the cellulose loss continuously increased with increasing NH_4Cl concentration, which indicates that higher dosages of NH_4Cl only caused more cellulose degradation. For the CYC, a noticeable inflection point emerged when 0.3 M NH_4Cl was used, which indicates that further increasing the NH_4Cl concentration had little influence on the CYC. Thus, the optimal NH_4Cl concentration for the pretreatment and enzymatic hydrolysis was approximately 0.3 M, at which point the CYC reached 85.5%.

pH values

The pH values of the pretreatment system were adjusted with HCl and NaOH to determine the effect of pH on the effect of pretreatment of ammonium chloride pretreatment system. The results are shown in Table 2. The generating rates of monosaccharides are accelerated by a low pH, so that amounts of furfural and 5-HMF are generated (Marzialetti *et al.* 2008). From pH 3 to pH 5, the SS yield, hemicellulose loss, and glucose and xylose contents in the PL changed slightly, which demonstrates that the pretreatment process was mostly affected by the NH_4Cl itself, not the pH value. During this process, large amount of ammonium ion in the solution could react with the

lignocellulosic materials by the ammonolysis of ester crosslinks of the xylan units and by cleaving the bonds linking between hemicellulose and lignin in LCC at high temperature (Wang *et al.* 1967). When the pH was less than 3, the major reaction was the hemicellulose hydrolyzed by hydronium ion when more hydrogen ions were present in the solution (similar to the dilute acid pretreatment) in addition to the chloride salts themselves, and more cellulose was degraded and dissolved into the PL. However, a notable influence on the CYC was caused by the pH values, which indicates that both the hydrogen ions and NH_4^+ had an important role in enzymatic scarification. Overall, it was suitable for the pretreatment when the pH value was adjusted to 4, and the CYC was increased to 92.0%. More importantly, these pH values could be reached using a small number of acids.

Table 2. Effect of pH Values on SS and PL[a]

pH	SS yield (%)	PL (mg/mL)		Hemicellulose loss (%)	CYC (%)
		Xylose	Glucose		
1	66.3	7.26	6.87	98.4	99.4
2	79.8	9.87	4.04	95.5	96.2
3	80.7	11.05	3.98	90.5	93.4
4	81.0	11.57	3.72	89.5	92.0
5	82.5	11.38	3.62	88.0	83.8
6	87.2	9.64	3.50	74.6	76.4

[a] Conditions: NH_4Cl 0.3 M, solid-to-liquid ratio 1:6, temperature 190 ºC, pretreatment time 20 min

Physical and Chemical Characteristics

The raw material and the SS (pretreated using 0.3 M NH_4Cl at 170 °C for 20 min) were detected by FTIR and XRD to explore their microscopic variation. The results are shown in Figs. 6 and 7. The chemical composition for the raw material and the pretreatment SS are shown in Table 3. After pretreated by NH_4Cl, most of the hemicellulose was hydrolyzed into the hydrolysate. With the degradation of the hemicellulose, the cellulose obviously increased from 40.39% to 63.29%. The relative content of lignin was increased slightly because of the degradation of hemicellulose and the solution of water extractions.

Table 3. Chemical Compositions of the Raw Material and SS

items	Cellulose，%	Hemicellulose，%	Lignin，%	Others，%
Raw material	40.39	31，75	29.68	1.72
SS	63.29	2.18	34.53	2.38

In Fig. 5, the crystallinities of the raw material and the SS were 51.5% and 63.3%, respectively. In connection with the removal of the amorphous substance of the hemicellulose degradation from the raw material, the SS mostly contained cellulose and lignin, and its crystallinity was obviously higher than that of the raw material. The crystal structure of cellulose was difficult to destroy in the NH_4Cl pretreatment process, and only a proportion of amorphous cellulose was degraded. However, the CYC was obviously improved by the pretreatment, which indicated that the crystallinity was not the main

factor that impacted the enzymatic hydrolysis process. The spectral assignments of FTIR were given in accordance with previous research (Yang *et al.* 2007; Zhang *et al.* 2016b).

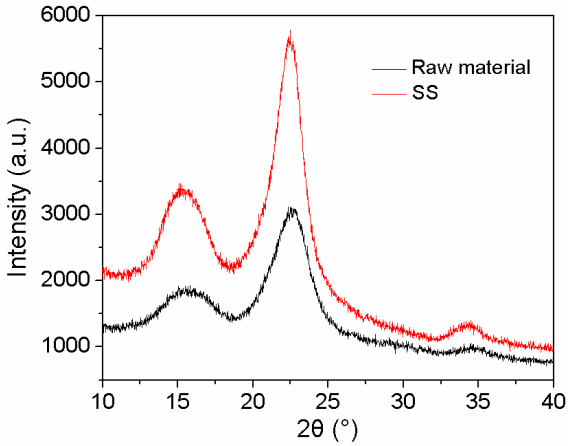

Fig. 5. XRD spectra of the SS and raw material

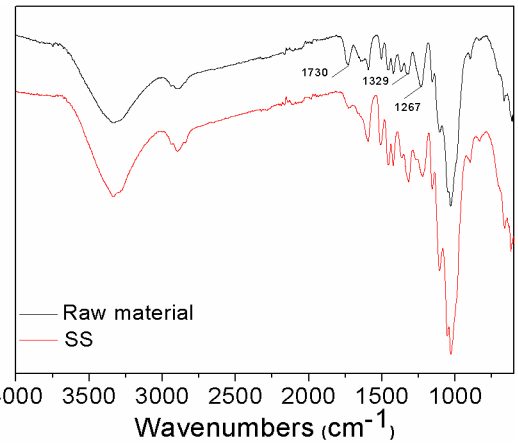

Fig. 6. FTIR spectra of the SS and raw material

In Fig. 6, the band at 3300 cm^{-1} to 2800 cm^{-1} was assigned to the stretching of asymmetric and symmetric methyl as well as methylene groups of cellulose, where the absorbance intensity in the SS was clearly stronger than that in the raw material. The band at 1710 cm^{-1} to 1730 cm^{-1} was the characteristic peak of hemicellulose, and it was assigned to carbonyl stretching in unconjugated ketones and carbonyl groups. The absorbance intensity in the SS was obviously weaker than that in the raw material, which demonstrates that a great amount of the hemicellulose was degraded in pretreatment. The band at 1329 cm^{-1} was assigned to condensed syringyl and guaiacyl rings, and the band at 1267 cm^{-1} corresponded to the signal of the guaiacyl ring. The raw material and the SS had strong absorption peaks in these two bands, which indicate that the pretreatment had little influence on lignin.

CONCLUSIONS

The NH_4Cl concentration, pH value, pretreatment time, and pretreatment temperature strongly affected the enzymatic hydrolysis of eucalyptus chips. In addition, longer pretreatment times and higher pretreatment temperatures were favorable for cellulose degradation, which caused a great amount of 5-HMF and furfural to be generated. NH_4Cl concentration had marginal impact on the SS yield and the cellulose degradation. Depending on the pH value, the NH_4Cl and hydrogen ions could combine to impact the pretreatment process. Thus, the optimal pretreatment conditions were 0.3 M NH_4Cl at 200 °C for 25 min.

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REFERENCES CITED

Anwar, Z., Gulfraz, M., and Irshad, M. (2014). "Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: A brief review," *J. Radiat. Res. Appl. Sci*. 7(2), 163-173. DOI: 10.1016/j.jrras.2014.02.003

Caffall, K. H., and Mohnen, D. (2009). "The structure, function, and biosynthesis of plant cell wall pectic polysaccharides," *Carbohyd. Res.* 344(14), 1879-1900. DOI: 10.1016/j.carres.2009.05.021

Ibrahim, M. M., Agblevor, F. A., and El-Zawawy, W. K. (2010). "Isolation and characterization of cellulose and lignin from steam-exploded lignocellulosic biomass," *BioResources*, 5(1), 397-418. DOI: 10.15376/biores.5.1.397-418

Kang, K. E., Park, D.-H., and Jeong, G.-T. (2013a). "Effects of NH_4Cl and $MgCl_2$ on pretreatment and xylan hydrolysis of *Miscanthus* straw," *Carbohyd. Polym.* 92(2), 1321-1326. DOI: 10.1016/j.carbpol.2012.10.019

Kang, K. E., Park, D.-H., and Jeong, G.-T. (2013b). "Effects of inorganic salts on pretreatment of *Miscanthus* straw," *Bioresour. Technol.* 132, 160-165. DOI: 10.1016/j.biortech.2013.01.012

Li, J., Henriksson, G., and Gellerstedt, G. (2007). "Lignin depolymerization/ repolymerization and its critical role for delignification of aspen wood by steam explosion," *Bioresour. Technol.* 98(16), 3061-3068. DOI: 10.1016/j.biortech.2006.10.018

Marzialetti, T., Valenzuela Olarte, M. B., Sievers, C., Hoskins, T. J. C., Agrawal, P. K., and Jones C. W. (2008). "Dilute acid hydrolysis of loblolly pine: A comprehensive approach," *Ind. Eng. Chem. Res.* 47(19), 7131-7140. DOI: 10.1021/ie800455f

Nishiyama, Y., Langan, P., and Chanzy, H. (2002). "Crystal structure and hydrogen bonding system in cellulose Iβ from synchrotron X-ray and neutron fiber diffraction," *J. Am. Chem. Soc.* 124(31), 9074-9082. DOI: 10.1021/ja0257319

Rollin, J. A., Zhu, Z., Sathitsuksanoh, N., and Zhang, Y. H. P. (2011). "Increasing cellulose accessibility is more important than removing lignin: A comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia," *Biotechnol. Bioeng.* 108(1), 22-30. DOI: 10.1002/bit.22919

Saeman, J. F. (1945). "Kinetics of wood saccharification - Hydrolysis of cellulose and decomposition of sugars in dilute acid at high temperature," *Ind. Eng. Chem.* 37(1), 43-52. DOI: 10.1021/ie50421a009

Segal, L. C., Creely, J. J., Martin Jr., A. E., and Conrad, C. M. (1959). "An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer," *Text. Res. J.* 29(10), 786-794. DOI: 10.1177/004051755902901003

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008). *Determination of Structural Carbohydrates and Lignin in Biomass* (NREL/TP-510-42618), National Renewable Energy Laboratory, Golden, CO.

Wang, P., Bolker, H., and Purves, C. (1967). "Uronic acid ester groups in some softwoods and hardwoods," *Tappi* 50, 123-124.

Yang, H., Yan, R., Chen, H., Lee, D. H., and Zheng, C. (2007). "Characteristics of hemicellulose, cellulose and lignin pyrolysis," *Fuel* 86(12-13), 1781-1788. DOI: 10.1016/j.fuel.2006.12.013

Zhang, W., Sathitsuksanoh, N., Simmons, B. A., Frazier, C. E., Barone, J. R., and Renneckar, S. (2016a). "Revealing the thermal sensitivity of lignin during glycerol thermal processing through structural analysis," *RSC Advances* 6(36), 30234-30246. DOI: 10.1039/C6RA00745G

Zhang, W., Sathitsuksanoh, N., Barone, J. R., and Renneckar, S. (2016b). "Enhanced enzymatic saccharification of pretreated biomass using glycerol thermal processing (GTP)," *Bioresour. Technol.* 199, 148-154. DOI: 10.1016/j.biortech.2015.08.141

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