EXTRACTION OF POLYMERS FROM ENZYME-TREATED SOFTWOOD

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In a biorefinery context it is an advantage to fractionate and extract different wood components in a relatively pure form. However, one major obstacle for efficient extraction of wood polymers (lignin, polysaccharides etc.) is the covalent lignin-polysaccharide networks present in lignified cell walls. Enzymatic catalysis might be a useful tool for a controlled degradation of these networks, thereby enhancing the extraction of high molecular weight polymers. In this work, a methanol-alkali mixture was used to extract two different wood samples treated with endoxylanase and gammanase, respectively. Wood chips were pretreated with alkali prior to enzymatic treatment to enhance the cell-wall accessibility to enzymes. Extractions were also carried out on non-enzyme-treated samples to evaluate the enzymatic effects. Results showed that the enzymatic treatment increased the extraction yield, with gammanase as the more efficient of the two enzymes. Furthermore, polymers extracted from xylanase-treated wood had a higher degree of polymerization than the reference.

Keywords: Pretreatment; Enzymatic treatment; Extraction; Lignocelluloses

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INTRODUCTION

Wood is by volume the most abundant renewable natural resource, available in natural forests and from plantations of fast growing trees, such as eucalyptus, Monterey pine, and acacia. The annual production of wood is therefore very high (Asikainen 2010). Other sources of lignocelluloses are various types of by-products from agriculture, such as straw, etc. These often have a similar chemical composition, *i.e.*, a composite type mixture of cellulose, hemicelluloses, and lignin (Sendich et al. 2009). Together, they represent an important renewable resource globally. Although wood can be used after minimal processing (for lumber, etc.), many applications require that the individual fibers are released (paper and board applications), or even that the individual polymers of lignocelluloses: cellulose, hemicelluloses, and lignin, are prepared in pure forms. For instance, cellulose derivatives such as cellulose acetate and carboxymethyl cellulose, as well as xylitol, and lignosulphonate require relatively pure starting materials. Purified hemicelluloses have fewer commercial applications than cellulose and lignin, but during the last years, interest in films and hydrogels based on hemicelluloses has increased (Karaaslan, et al. 2010; Mikkonen et al. 2009; Sternemalm et al. 2008). Except for making the anionic polymer lignosulphonate, which is used in making concrete, lignin is

mainly used as fuel and as raw material for manufacture of vanillin. On an experimental level lignin has also been tested as a gluing substance and as raw material for manufacture of carbon fibers (Akhtar et al. 2009; Gosselink et al. 2004; Pereira et al. 2007).

With increasing prices for petroleum and a growing concern over environmental effects of large-scale use of non-renewable resources, it can be predicted that the market for purified wood polymers will be increased. Therefore, there is a need for efficient industrial methods for preparation of such materials from wood. Selective extraction of wood materials may be a good way, and organic solvents can be used for this purpose. Alcohols have been used in the past to split lignocelluloses into their components; however the need of complicated equipment to afford elevated pressure at high temperature has discouraged implementation of these processes (Muurinen 2000). Hot water extractions of hemicelluloses have also been studied from both hardwood (Casebier et al. 1969; Leschinsky et al. 2008; Tunc et al. 2008) and softwood species (Song et al. 2008; Willfor et al. 2003). During hot water extraction the pH drops due to the formation of acetic acid from hemicelluloses, leading to partial hydrolysis of the wood polysaccharides in a process known as *autohydrolysis*. Thus, relatively moderate alkaline solutions, as compared to kraft process, have been used in order to limit polysaccharide degradation (van Heiningen 2006). These conditions, however, lower the yield of extraction of hemicelluloses and result in contamination of the lignin.

One possible explanation for such effects is the recent finding that lignin in wood is covalently cross-inked with different wood polysaccharides into large networks (Lawoko et al. 2005). This might be an obstacle for quantitative extraction of hemicelluloses (Tunc et al. 2010). One way to increase the yield of extractions should then be by separating the polymers by specific cleavage of strategic covalent bonds. This requires specific catalysis, and nature provides such catalysts in the form of enzymes. Several such enzymes have been cloned and expressed on an industrial scale for applications such as laundry detergents (Horikoshi 1999) and bleaching of pulps (Simeonova et al. 2007; Viikari et al. 1991). However, one problem with such a strategy is that wood structure is so compact that molecules as large as enzymes cannot penetrate into the cell wall (Blanchette et al. 1997). On the other hand, treating the wood with sodium hydroxide/sodium sulfide solution can "open up" the structure and allow for enzymatic treatment (Wang et al. 2011).

The present work was aimed at understanding the effect of enzymatic treatment of wood on the results of extractions with organic solvents, with the possibility of obtaining better yields of high molecular weight polymers including hemicelluloses and lignin.

EXPERIMENTAL

Materials

Norway spruce (*Picea abies*) chips (approx. dimensions 20 mm, 10 mm, and 3 mm) were kindly supplied by Holmen Paper AB, Hallstavik Sweden. The chemical composition of the chips is shown in Table 1. Pulpzyme HC: a monocomponent endo-1,4-β-D xylanase (Roncero et al. 2003) and Gammanase: a culture filtrate rich in 1,4-β-D-Mannan mannanohydrolase obtained from *Aspergillus niger* that also contained beta-

glucuonidase activity (Redgwell et al. 2005) were kind gifts from Novozyme, Denmark. All other chemicals were of analytical grade.

Methods

Alkaline pretreatment

Norway spruce chips were pretreated in a laboratory circulation digester in a manner analogous to the traditional kraft pulping. Wood chips (1 kg o.d.) were first steamed for 5 min under 1.5 MPa pressure. Subsequently, white liquor with 60% sulfidity and 35 g/L sodium hydroxide concentration was added with a liquor-to-wood ratio of 4:1. The temperature was raised by 1 °C/min from 100 °C to the desired cooking temperature of 150 °C and kept constant for 30 min. Thereafter, the wood chips were washed for about 12 h at a flow of 1.8 dm^3/min of deionized water. Alkaline pretreated chips were disintegrated according to ISO 5263-1:2004 at approx. 15 g dry material per liter of deionized water and then defibrillated at 2 bars water pressure in a water jet defibrillator from Nordiska Armaturfabriken (Sweden) with 15 mm perforations.

Enzymatic treatment

Enzymatic treatment of pretreated wood was performed as follows: For Pulpzyme HC (xylanase) the wood sample was suspended in a 20 mM phosphate buffer pH 7 at 40 °C and incubated for 24 h (Roncero et al. 2003) with the enzyme dosage of 1000 EXU/g pulp (1 mL/g). For gammanase treatment the buffer was 50 mM sodium acetate pH 5, the incubation was carried out at 60 °C for 24 h, and the enzyme dosage was 1000 VHCU/g (1 mL/g). In both cases the pulp concentration was 3%, and the enzymatic treatment was terminated by incubation at temperature >90 °C for 15 min.

Extraction

10 g o.d. mass of enzyme-treated wood was extracted with a mixture of 50% w/w methanol, containing about 5% alkali charge on wood calculated as sodium hydroxide (Muurinen 2000). Alkaline pre-treated wood, followed by defibrillation and without any enzymatic treatment, was extracted in parallel as a reference material (Fig.1). Extractions were carried out in 5 L stainless steel autoclaves that rotated for 2 h in a heated mineral oil bath at 130 °C. Subsequently the autoclaves were cooled in running water and emptied, followed by rinsing with some deionized water. The material was filtered with wire cloth and thoroughly washed. The residues were kept at 4 °C, whereas the obtained extracts were stored at room temperature.

Analysis

The amount of material extracted was measured gravimetrically by drying aliquots overnight at 105 °C. Klason lignin content was determined by acid hydrolysis according to TAPPI T222 om-83. Acidic hydrolysate from klason lignin determination was analyzed for sugar composition. Analysis of sugar contents was performed on a Dionex ICS-3000 High Performance Anion Exchange Chromatography (HPAEC) equipped with pulsed amperometric detector using a Dionex PA1 column. Five monomeric sugars: arabinose, galactose, glucose, xylose, and mannose, were used to prepare calibration standards with five different concentrations.

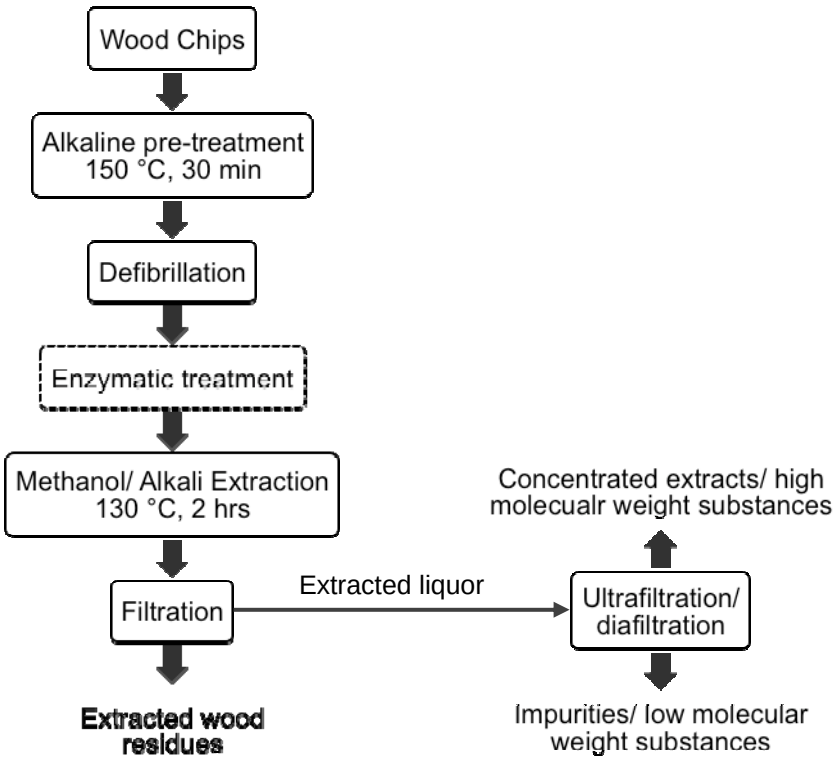

Fig. 1. Scheme representing the isolation of high molecular weight polymers from wood chips via enzymatic treatment. For controls, extractions were carried out on non-enzyme-treated samples.

All the obtained extracts were ultrafiltrated and diafiltrated with a 1000 Da regenerated cellulose membrane in a pressurized stirred cell (Millipore, XFUF 047 01), to concentrate and to increase the purity of the extracted polymers. The concentrated samples were freeze-dried and analyzed for sugar composition with HPAEC. The average molar mass (M_w) of the dissolved polymers was determined by high performance Size Exclusion Chromatography (SEC) equipped with a refractive index detector. SEC was calibrated with polyethylene oxide/ polyethylene glycol standards with molecular weights in the range of 1500 to 250 000 Da. Extracted residues were delignified by repeated treatment with aqueous sodium chlorite at 70 °C (Ahlgren et al. 1971). Lignin-free holocellulose was used to determine the viscosity according to the ISO 5351:2010 standard.

RESULTS AND DISCUSSION

Wood chips were pretreated with alkali, prior to enzymatic treatment, to "open up" their compact structure. Alkaline pretreatment of wood chips at 150 °C for 30 min caused a loss of about 30% of lignocelluloses present in wood. This pretreated wood contained 30% of glucomannan, 93% of xylan, and 47% of lignin, based on the amounts present in the original wood (Table 1). However, removal of a part of these lingocelluloses created the gap needed for enzymes to penetrate and attack the remaining

polymers, as was evident in the subsequent enzymatic treatment and also was consistent with published work (Wang et al. 2011).

Table 1. Lignocellulosic Composition of Spruce Wood & Pretreated Wood (% on wood)

Compounds	Spruce chips (% by wt.)	Chemically pretreated wood (% by wt.)
Arabinan	1.2	0.9
Galactan	1.7	1.0
Glucan	44.5	43.7
Xylan	5.7	5.3
Mannan	11.9	3.6
Klason lignin	28.5	13.4
Glucomannan*	14.9	4.5

*Calculated with the ratio of glucose:mannose as 1:4.

Pretreated wood was then treated with enzymes to improve the extractability of lignocelluloses. A mixture of methanol and alkali was used to extract lignocellulosic components. The extraction yield with this mixture was, however, much lower than that of traditional organosolv pulping performed at 160 to 180 °C (Muurinen 2000). A temperature of 130 °C was used in this study in order to lessen depolymerization, which is incurred at high temperature.

Results showed that enzymatic treatment enhanced the amount of extracted material to a certain extent, as presented in Fig. 2. Different types of enzymes do however have different efficacy; a significantly larger amount of material was extracted from the gammanase-treated material than the reference, whereas the difference was lower for the xylanase-treated sample. Xylanase treatment enhanced the extraction by 2% (w/w), whereas gammanase treatment enhanced it by approximately 9% (w/w).

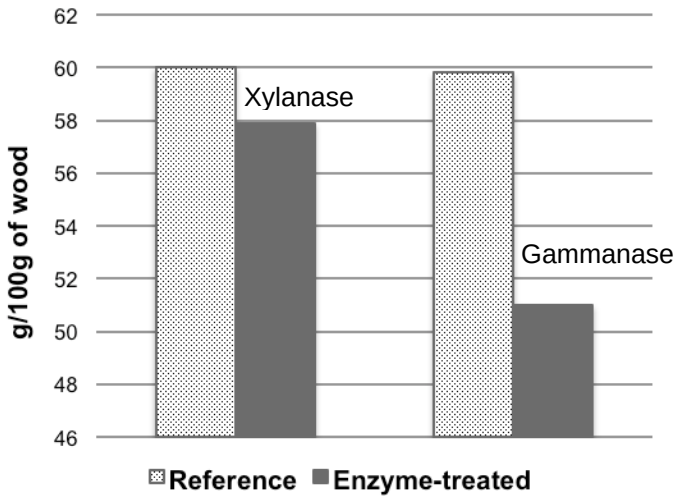

Fig. 2. Extracted residues of enzyme-treated samples with respect to references. The data show that significantly more material could be extracted from wood after hemicellulase treatment, and that the effects were stronger for the gammanase-treated material.

Endoxylanse specifically cleaved the xylan chains and improved the extraction of xylan as well as other components (Fig. 3a). Gammanase attacked the glycosidic linkages of glucomannan, resulting in a better extraction of glucomannan (Fig. 3b). Interestingly, the extraction of lignin and glucan was also enhanced by gammanase treatment. Under all circumstances, the fact that non-glucomannan wood polymers become easier to extract following mannanase treatment of the wood is in line with previous reports on ultrastructural arrangement of wood polymers (Salmen et al. 1998). Similarly, the enhanced extraction of lignin from gammanase-treated wood may be explained by the existence of covalent linkages of lignin with glucomannan.

Each enzymatic treatment did increase the extraction of both glucomannan and xylan, as shown in Fig. 3. This could be due to the fact that the two hemicelluloses are morphologically located partly in layers. Thereby, increased extractability of one hemicellulose may increase the extraction of the other hemicellulose. Also, viscosity measurements on residues showed no degradation of cellulose caused by enzymes, as the viscosity of all the samples was around 900 g/mL, similar to the references.

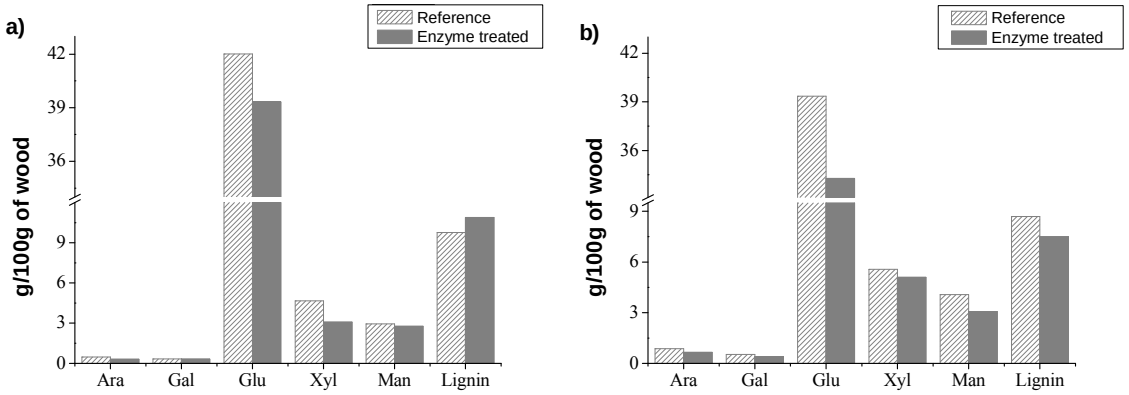

Fig. 3. Amount and compositions of lignocelluloses in the extracted residues of enzyme-treated wood, in comparison with references a) xylanase-treated b) gammanase-treated

The extracted material was purified from low molecular weight substances by ultrafiltration and dialfiltration. Klason lignin analysis of the dried extracts showed that each dried sample contained about 70% lignin.

All of the dried extracts were analyzed with Size Exclusion Chromatography to compare the average molar mass (M_w) of the extracted components. SEC chromatograms in Fig. 4 showed that material extracted from xylanase treated wood had a clearly higher molecular weight as compared to the material extracted from the reference sample. Material obtained from the sample treated with mannanase, however, had a molecular weight in the same range as reference, which could be due to the excessive degradation of glucomannan by mannanse treatment.

The exact reasons for the observed differences in extract yields and molar mass when different enzymes were applied are still unclear. However several hypotheses such as differences in enzyme efficacy, effects of ultrastructure arrangement of polymers on enzyme activity, as well as effects of the relative amounts of available substrate and their distribution in the cell wall, still remain to be investigated.

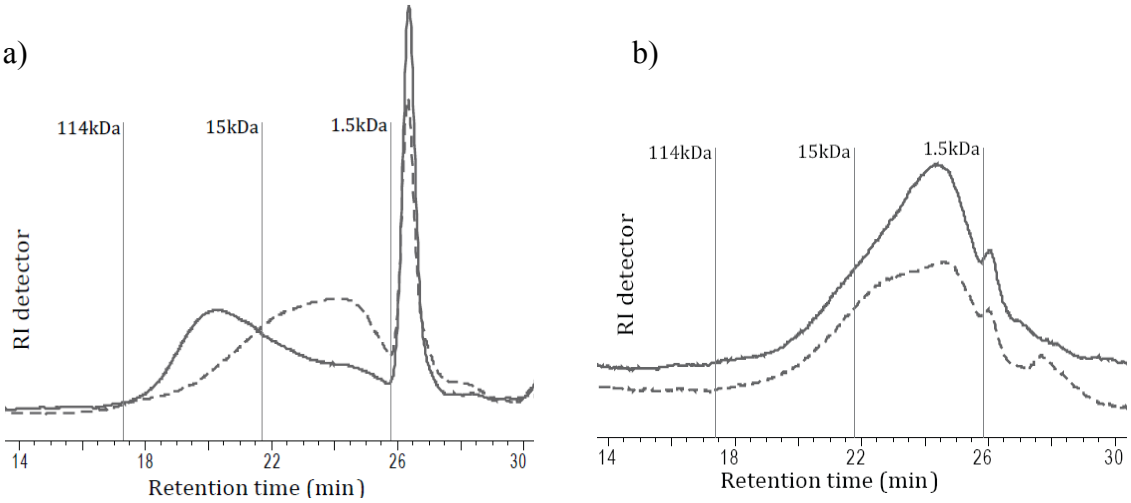

Fig. 4. SEC chromatograms of concentrated extracts from the enzyme-treated wood and the references. a) xylanase-treated b) gammanase-treated. - - - - reference, —— enzyme-treated.

CONCLUSIONS

The study has shown that the application of enzymes may be a promising approach to improve both extraction yield as well as extraction of high molar mass material. More specifically, the application of xylananse led to enhanced extraction of high molar mass material, whereas gammanase treatment improved the yield of lower molar mass material. However, a pretreatment step is necessary to enable the required enzyme action.

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REFERENCES CITED

Ahlgren, P. A., and Goring, D. A. I. (1971). "Removal of wood components during chlorite delignification of black spruce," *Canadian Journal of Chemistry* 49(8), 1272-1275.

Akhtar, T., Lutfullah, G., and Nazli, R. (2009). "Synthesis of lignin based phenolic resin and its utilization in the exterior grade plywood," *J. Chem. Soc. Pakistan* 31(2), 304-308.

Asikainen, A. (2010). "Availability of wood biomass for biorefining," *Cell. Chem. Technol*. 44(4-6), 111-115.

Blanchette, R. A., W. Krueger, E., Haight, J. E., Masood, A., and Akin, D. E. (1997). "Cell wall alterations in loblolly pine wood decayed by the white-rot fungus, *Ceriporiopsis subvermispora*," *Journal of Biotechnology* 53(2-3), 203-213.

Casebier, R. L., Hamilton, J. K., and Hergert, H. L. (1969). "Chemistry and mechanism of water prehydrolysis of southern pine wood," *Tappi* 52(12), 2369-2377.

Gosselink, R. J. A., Snijder, M. H. B., Kranenbarg, A., Keijsers, E. R. P., de Jong, E., and Stigsson, L. L. (2004). "Characterisation and application of NovaFiber lignin," *Ind. Crop. Prod*. 20(2), 191-203.

Horikoshi, K. (1999). "Alkaliphiles: Some applications of their products for biotechnology," *Microbiol. Mol. Biol. R*. 63(4), 735-750.

Karaaslan, A. M., Tshabalala, M. A., and Buschle-Diller, G. (2010). "Wood hemicellulose/chitosan-based semi-interpenetrating network hydrogels: Mechanical, swelling and controlled drug release properties," *BioResources* 5(2), 1036-1054.

Lawoko, M., Henriksson, G., and Gellerstedt, G. (2005). "Structural differences between the lignin-carbohydrate complexes present in wood and in chemical pulps," *Biomacromolecules* 6(6), 3467-3473.

Leschinsky, M., Zuckerstatter, G., Weber, H. K., Patt, R., and Sixta, H. (2008). "Effect of autohydrolysis of *Eucalyptus globulus* wood on lignin structure. Part 1: Comparison of different lignin fractions formed during water prehydrolysis," *Holzforschung* 62(6), 645-652.

Mikkonen, K. S., Heikkinen, S., Soovre, A., Peura, M., Serimaa, R., Talja, R. A., Helen, H., Hyvonen, L., and Tenkanen, M. (2009). "Films from oat spelt arabinoxylan plasticized with glycerol and sorbitol," *J. Appl. Polym. Sci.* 114(1), 457-466.

Muurinen, E. (2000). "Organosolv pulping - A review and distillation study related to peroxyacid pulping," University of Oulu, Finland.

Pereira, A. A., Martins, G. F., Antunes, P. A., Conrrado, R., Pasquini, D., Job, A. E., Curvelo, A. A. S., Ferreira, M., Riul, A., and Constantino, C. J. L. (2007). "Lignin from sugar cane bagasse: Extraction, fabrication of nanostructured films, and application," *Langmuir* 23(12), 6652-6659.

Redgwell, R. J., Schmitt, C., Beaulieu, M., and Curti, D. (2005). "Hydrocolloids from coffee: Physicochemical and functional properties of an arabinogalactan-protein fraction from green beans," *Food Hydrocolloid* 19(6), 1005-1015.

Roncero, M. B., Torres, A. L., Colom, J. F., and Vidal, T. (2003). "Effect of xylanase on ozone bleaching kinetics and properties of eucalyptus kraft pulp," *J. Chem. Technol. Biot*. 78(10), 1023-1031.

Salmen, L., and Olsson, A. M. (1998). "Interaction between hemicelluloses, lignin and cellulose: Structure-property relationships," *J. Pulp Pap. Sci.* 24(3), 99-103.

Sendich, E. D., and Dale, B. E. (2009). "Environmental and economic analysis of the fully integrated biorefinery," *GCB Bioenergy* 1(5), 331-345.

Simeonova, G., Sjodahl, R., Ragnar, M., Henriksson, G., and Lindström, M. E. (2007). "On the effect of a xylanase post-treatment as a means of reducing the yellowing of bleached hardwood kraft pulp," *Nord. Pulp Pap. Res. J*. 22(2), 172-176.

Song, T., Pranovich, A., Sumerskiy, I., and Holmbom, B. (2008). "Extraction of galactoglucomannan from spruce wood with pressurised hot water," *Holzforschung* 62(6), 659-666.

Sternemalm, E., Höije, A., and Gatenholm, P. (2008). "Effect of arabinose substitution on the material properties of arabinoxylan films," *Carbohydrate Research* 343(4), 753-757.

Tunc, M. S., Lawoko, M., and van Heiningen, A. (2010). "Understanding the limitations of removal of hemicelluloses during autohydrolysis of a mixture of southern hardwoods," *BioResources* 5(1), 356-371.

Tunc, M. S., and van Heiningen, A. R. P. (2008). "Hemicellulose extraction of mixed southern hardwood with water at 150 degrees C: Effect of time," *Industrial & Engineering Chemistry Research* 47(18), 7031-7037.

van Heiningen, A. R. P. (2006). "Converting a kraft pulp mill into an integrated forest biorefinery," *Pulp Pap-Canada* 107(6), 38-43.

Viikari, L., Sundquist, J., and Kettunen, J. (1991). "Xylanase enzymes promote pulp bleaching," *Pap. Puu - Pap. Tim.* 73(5), 384-389.

Wang, Y., Lindström, M. E., and Henriksson, G. (2011). "Mild alkaline treatment activates spruce wood for enzymatic processing: A possible stage in biorefinery processes," *BioResources* 6(3), 2425-2434.

Willför, S., Sjöholm, R., Laine, C., Roslund, M., Hemming, J., and Holmbom, B. (2003). "Characterisation of water-soluble galactoglucomannans from Norway spruce wood and thermomechanical pulp," *Carbohydrate Polymers* 52(2), 175-187.

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