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Stabilisation of polysaccharides during alkaline pretreatment of wood combined with enzyme-supported extractions in a biorefinery

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Specific enzymes have been demonstrated to increase the possibilities for extracting wood polymers. Enzymatic treatment requires an open wood structure, which was achieved by extended impregnation of the wood. However, some of the hemicelluloses, primarily glucomannan, and lignin were lost during the impregnation. To improve the carbohydrate yield, three glucomannan modification agents: sodium borohydride, polysulphide and anthraquinone, were used, which increased the yields of the impregnated materials from 76.6% to 89.6%, 81.3% and 80.0%, respectively. Through the use of additives, most of the glucomannan could be retained in the wood while still allowing the enzymes to penetrate the wood and attack the polymers. The additives also increased the extraction yield from 9 to 12% w/w wood. Gamanase treatment prior to the extraction increased the extraction yield to 14%. Of the three stabilising agents, sodium borohydride was the most efficient, providing the highest extraction yields.

Keywords: extended impregnation, peeling reaction, sodium borohydride (NaBH₄), polysulphide (PS), anthraquinone (AQ), extraction, biorefinery.

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INTRODUCTION

Due to concerns regarding climate change and dwindling natural resources, renewable raw materials must be found to replace petroleum. The biomass materials described herein are promising candidates given their natural abundance.^[1] Biomass materials are often complex in composition; plant materials, for example, contain three major components: cellulose, hemicellulose, and lignin. These three components all have interesting properties. *Cellulose* is a linear polysaccharide with a high degree of polymerisation that can be used in various types of materials. Cellulose can also be derivatised or regenerated in various ways to create new materials that are useful in a variety of applications.^[2] *Hemicelluloses* are the second most abundant polysaccharides in nature, primarily found as amorphous heteropolysaccharides, which can be used in various types of films and barriers, and for chemicals.^[3] It is also available as substrates for producing valuable industrial products such as ethanol, xylitol and butandiol.^[4] *Lignin* is the third main component in wood and is not a polysaccharide but is a cross-linked polymer of phenylpropanoid units joined through ether or carbon-carbon bonds. One form of modified lignin, lignosulphonates, find many commercial uses, such as dispersing agents in concrete, antioxidants in cosmetics, and as an asphalt emulsifying agent.^[5-6] Lignin can also be used for producing carbon fibres, as a board binder, in batteries and in solid fuels, etc.^[7] So, if a chemical process uses only one of these three components, and the others become waste; the process then often becomes uneconomic. In traditional chemical pulping of wood, cellulose is the main product whereas most of the valuable hemicelluloses are lost in the black liquor and burned together with lignin during the chemical recovery cycle. In the kraft- and sulphite processes, various low value added bi-products are also produced, such as tall oil, turpentine and

lignosulphonates. Therefore, novel biorefinery processes for effective separation of wood components would be of considerable interest in order to produce a variety of value added products from each component. In the compact wood structure, lignin crosslinks the polysaccharides and large lignin carbohydrate complex (LCC) networks are formed.^[8] Thus, separating the lignocellulosic components without requiring intense chemical and energy consumption is not an easy task. Furthermore, the extensive use of chemicals and energy results in damage to and loss of the wood components. Enzymes were introduced to untie the lignin polysaccharide networks prior to separating the wood components.^[9] The enzymes have high substrate specificity and require relatively mild working conditions. However, the compact structure of wood inhibits the large enzyme molecules to efficiently attack the substrate polymers.^[10] A pretreatment method, referred to as extended impregnation, was used to open the wood structure that allowed the large enzyme molecules to penetrate. Extended impregnation was based on traditional kraft pulping process, however, much milder conditions were applied than kraft process. Kraft pulping process is a well-established method for chemical pulping and is easy to use in large-scale application. Moreover, white liquor can easily be produced at a large scale for a low price in processes integrated in chemical recovery system of kraft mills. Extended impregnation of wood with white liquor enables the enzymes to penetrate the wood structure and act as specific LCC-network-dissolving catalysts. An extraction process was performed on the hemicellulases treated materials and the result indicated that all enzymatic treatments increased the extraction yield; a glucomannanase-rich culture filtrate was the most efficient. The polymers extracted from the xylanase-treated material exhibited a higher degree of polymerisation than the non-enzymatically treated controls.^[11] Therefore, the possibilities to develop biorefinery

processes based on enzymatic treatment of wood biomass, which were not only limited on pulp product, appeared to be promising. However, the valuable glucomannan is significantly degraded during the pretreatments. The sensitivity of this polysaccharide is likely due to a “peeling” reaction that degrades the glucomannan from the reducing end (Fig. 1). Arabinoxylans are less sensitive to this type of degradation since the presence of a substituent, arabinose, in the 3-position favours a direct β -elimination from the open aldehyde form as illustrated in Fig. 1. Thereby, a stabilisation of the polysaccharide chain takes place that prevents the peeling reaction to proceed.^[12] To improve on this concept, some modifications must be made to protect the peeling reaction from alkaline attack.

The glucomannan is stabilised against alkali degradation when the carbonyl groups, responsible for peeling, are reduced or oxidised to relatively stable end groups preventing further peeling reactions.^[13] Three common chemicals—sodium borohydride (NaBH_4), polysulphide (PS) and anthraquinone (AQ)—can be used to protect the reducing end of the glucomannan from the peeling reaction.^[14-15] Peeling reaction is initiated by the carbonyl group in the end unit of the carbohydrate chains (Fig. 1). Borohydride is a powerful reducing agent that reduces the carbonyl group to hydroxyl groups which prevents the end-wise depolymerisation of the glucomannan (Fig. 2).^[16] For the polysulphide additive, the addition of elemental sulphur to the cooking liquor generates polysulphides represented as S_n in which $n = 2-8$. In the stabilisation reaction the enediol form of the reducing end-group, which is in equilibrium with the corresponding open aldehyde form, is believed to be oxidised to a diketo structure and transferred via a benzilic acid rearrangement to the aldonic acid (Fig. 2).^[17] The carboxylic group is alkali-stable, allowing stabilisation against peeling.

This protection of the reactive end group could inhibit the peeling reaction and enhance the yield by leaving more carbohydrates in the wood. In the 1970s, many researchers started investigating anthraquinone (AQ) and its derivatives as pulping additives.^[18] The chemistry of AQ begins with a rapid oxidation of the reducing end-groups in the glucomannan, as depicted in Fig. 2.^[19] In this reaction, anthrahydroquinone (AHQ) was formed and reacted with the quinone methide intermediates formed in the lignin under alkali conditions. The adduct between the AHQ and the quinone methide decomposes with the formation of the AQ, and the β -O-4 linkage is simultaneously cleaved to form coniferyl alcohol and new phenolic end-groups.^[20] Adding relatively small quantities of AQ (0.04–0.1%) to the cooking liquor could accelerate the delignification rate providing less time than required for the carbohydrate peeling reaction,^[21] which would stabilise the reducing end-groups of carbohydrates via the redox cycle. The pulp yield can also be increased by inhibiting the peeling reaction.

In this study, we examine wood pretreatments using sodium borohydride, polysulphide and anthraquinone, compare their effects on the yield, and evaluate their influence over the enzymatic treatment and extraction processes.

EXPERIMENTAL

Materials

The Norway spruce (*Picea abies*) wood chips used were supplied by Hallsta Pappersbruk, Sweden. The enzymes used were Novozym 342, a cellulolytic culture filtrate primarily containing endoglucanase, cellobiohydrolase, β -glucosidase and xylanase with other hemicellulases,^[22-23] gamanase, which primarily contains 1,4- β -

D-Mannan mannanohydrolase obtained from *Aspergillus niger* and β -glucuronidase.^[24]

The enzymes were kindly donated by Novozymes, Denmark. All other chemicals were of analytical grade.

Methods

Wood pretreatment

Norway spruce (*Picea abies*) chips (1 kg o.d. weight) were impregnated with white liquor (a solution of Na₂S and NaOH) based on the traditional kraft cooking process. The impregnation conditions are provided in Table 1 and this treatment is referred to as extended impregnation (EI) herein because of the low treatment temperature as compared to kraft cooking. The treatment time for EI was longer than that required for impregnation during the typical kraft process. In modern kraft cooking processes, part of the black liquor is recycled to the impregnation stage. The black liquor still contains high amount of hydrogen sulfide ions but low hydroxide ion concentration. Thus, high sulfidity was used in the EI to simulate the conditions of pulp mill operation. Moreover, high liquor to wood ratio can help the impregnation of the chemicals to the wood chips. Extended impregnation performed by adding NaBH₄, elemental sulphur (PS) or anthraquinone (AQ) is referred to as a modified extended impregnation (Table 1).

The impregnation was performed using a pilot-scale circulation digester. Chips were fed into the digester tank, and the alkaline liquor was circulated after steaming the chips for 5 min. The temperature was increased from 100 °C at a rate of 1 °C/min until reaching the desired temperature, which was maintained for 2 h. After this alkaline treatment, the wood chips were washed with deionised water with a flow

rate of 1.8 dm³/min for more than 12 hours to remove the alkaline residue. The pretreated wood chips were disintegrated with 50,000 revolutions in a disintegrator designed according to ISO 5263-1:2004. The sample ratio in the disintegrator was approximately 30 g dry material to 2 dm³ deionised water.

Enzymatic treatment

These four types of samples were incubated with Novozym 342 to determine whether the modified extended impregnation samples could be opened sufficiently for the enzyme to penetrate the wood and attack the polymers. The incubation process was performed using 5 mg (dry weight) wet samples suspended in a 20 mM sodium phosphate buffer (pH 7) with 5 µL Novozym 342 (90 EGU/g) at 40 °C for 5 h in a final volume of 1 mL. The incubation process was performed in a Thermomixer comfort (Eppendorf, Hamburg, Germany) with shaking at 600 rpm. The reaction was terminated by increasing the temperature above 90 °C.

A large-volume enzymatic treatment was performed with gamanase prior to the extraction. A 10 g (o.d. weight) aliquot of each sample was incubated with 300 mL 50 mM (pH 5) sodium acetate buffer at 60 °C for 24 h. The enzyme dosage was 1000 VHCU/g (1 mL/g). The incubation processes were performed in a large water bath, and the enzymatic treatments were terminated by incubating at temperatures above 90 °C for 15 min and filtering through a Buchner funnel.

Extraction experiments

The extractions were conducted in 2-L stainless steel autoclaves rotating in a high-pressure vessel filled with polyethylene glycol as the heating medium. The four enzyme-treated samples were extracted together with the non-enzyme-treated

samples as references. A 10 g o.d. aliquot of each sample was extracted with a mixture of 50% w/w methanol containing 5% w/w alkali charge on wood.^[25] The liquid-to-wood ratio was 10:1. The material was heated at 130 °C for two hours. Thereafter, the autoclaves were quenched in a cold-water bath for 30 minutes. The liquid containing the dissolved solids was separated from the wood via vacuum filtration using a wire cloth (mesh size: 71 microns) and washed with deionised water. Aliquots of the residual wood were heated at 105 °C to determine the quantity of material extracted. The liquids containing the dissolved solids were lyophilised.

Analysis

Reducing sugar analysis

The reducing sugars were analysed according to the method described by Miller^[26] in which a colour reaction is used to determine the reducing sugar concentration. The DNS reagent was composed of 1% dinitrosalicylic acid, 1% NaOH, 10% NaK tartrate, 0.05% Na₂SO₃ and 0.2% phenol dissolved in water. 1 mL of the sample was mixed with 1 mL DNS reagent and centrifuged in a table centrifuge for three minutes at 14,100 rpm. After centrifugation, 1 mL of the supernatant was transferred to a new tube and boiled for 5 min. Glucose standards with known concentrations from 0.5 mM to 4 mM were simultaneously processed in the same manner. Ice water was used to cool the samples, and a Cary 100 UV/VIS spectrophotometer (Varian, Palo Alto, CA, USA) was used to record the absorbance of both the glucose standards and samples at 575 nm. The concentrations of reducing sugar released can be calculated using the glucose standard curve.

Klason lignin content

The Klason lignin content was determined by hydrolysing the samples according to SCAN-CM 71:09. The hydrolysates were cooled and filtered with a 1.6- μ m GF/A filter and then washed with 5 mL hot and 5 mL cold water. The insoluble material remaining on the filter was analysed gravimetrically for Klason lignin after drying overnight at 105 °C.

Carbohydrate composition

The carbohydrate composition of the filtrates after acid hydrolysis (SCAN-CM 71:09) was analysed via high-performance anion-exchange chromatography (Dionex, Sunnyvale, CA, USA) with a pulsed amperometric detector (HPAEC-PAD) and a CarboPac PA-1 column. To analyse the monosaccharides, the system was equilibrated for 7 min with 260 mM sodium hydroxide and 170 mM sodium acetate followed by 100% ultrapure water for 6 min. Only ultra-pure water was used as an eluent during the sample injection at a flow rate of 1 mL/min. A solution of 300 mM NaOH was added to the column effluent prior to the PAD cell at a flow rate of 0.5 mL/min. The data were processed using Chromeleon 7.1 software.

Molar mass distribution

The molar mass distribution of the lyophilised extracts was determined via size exclusion chromatography in a system equipped with a Rheodyne 7725i (Rohnert Park, CA, USA) manual injector, a Waters 515 HPLC pump (Milford, MA, USA) and three TSK-gel columns (Tosoh Bioscience, Tokyo, Japan) coupled in series, two G3000PWs (7.5 $\times$ 300 mm, 10- μ m particle size) and one G4000PW (7.5 $\times$ 300 mm, 17- μ m particle size). For detection, a Waters 2487 dual-wavelength absorbance detector (Milford, MA, USA) and a Waters 410 refractive index (RI) detector (Milford, MA, USA) were used.

The eluent system contained 10 mM sodium hydroxide in ultra-pure laboratory-grade Milli-Q water. A 20- μ L sample was injected, and both the UV absorbance at 280 nm and RI were recorded. The columns were calibrated with pollulan standards with specific molecular weights ranging from 320 to 400,000 Da. The peaks were integrated and quantified using the supplied Millenium 2 software.

RESULTS AND DISCUSSION

The pretreatment was performed by impregnating the wood either without additives (extended impregnation) or by the modified method in which sodium borohydride, polysulphide or anthraquinone, was added (modified extended impregnation). The samples were analysed for their yield and their lignin and carbohydrate contents, as provided in table 2. All wood composition data was based on original o.d. spruce samples.

The samples treated with any of the modified extended impregnation exhibited considerably higher yields; wherein borohydride had the highest yield. The Klason lignin analyses indicated that all treated samples had similar lignin contents (Table 2); therefore, the difference in yield is likely due to differences in the carbohydrate content. In fact, the monosaccharide content was higher in the samples that underwent the modified extended impregnation with the sodium borohydride-treated sample exhibiting the highest sugar content (Table 2). The largest effect was noted with mannose, indicating that the increased yield is largely due to the preservation of the glucomannans by the modified treatments.

Of the modifications, the sodium borohydride reducing agent was more efficient than the polysulphide and anthraquinone oxidising agents (Table 2). Oxidation of carbonyl end groups creates carboxylic acids, which can also create enols that initiate

the peeling reaction (Fig 1), however, at a very low rate due to resonance stabilization of the carboxylic acid. The reduced structure, on the other hand, contains no double bond to initiate the peeling reaction. Thus, reduction is likely a very efficient method for preventing the peeling reaction, as previously suggested.^[27] The degradation of the polysaccharides under alkaline conditions, such as those that occur during kraft pulping, is believed to proceed through two main reactions: the slow alkaline hydrolysis within a chain to produce free reducing ends on the polysaccharide, and the peeling reaction, which is a step-wise removal of the monosaccharide residues from the reducing end (Fig. 1). The oxidative treatments have the advantage that the oxidation can be carried out in the same stage as the alkaline hydrolysis, as a type of “sealing”, whereas the sodium borohydride treatment is a pretreatment separated from the alkaline hydrolysis. This suggests that the alkaline hydrolysis plays a less important role in our system for degrading hemicellulose. Therefore, the wood after modified extended impregnation have high hemicellulose content, especially glucomannan (Table 2). In addition, the absolute Klason lignin values were more or less identical, which indicates that the additives did not influence the Klason lignin content. In traditional kraft pulping of softwood, the initial lignin dissolution occurs rapidly at temperatures below 140 °C and dissolves about 20% of lignin. During the extended impregnation at 110 °C a similar amount of lignin was already lost, thus no acceleration of the delignification rate by the additives was observed.

The extended impregnation process opens the compact wood structure to allow enzyme penetration has already been demonstrated in an earlier work.^[9] However, the aim of the modified extended impregnation was to stabilise the glucomannan content, allowing more materials to stay in the wood. Thus whether large enzymes

could also penetrate the wood remained uncertain. The initial investigations of the modified extended impregnated materials were also performed with Novozym 342, a commercially available cellulolytic culture filtrate that can degrade most cell wall polysaccharides. The released reducing sugar was measured to determine whether the enzymes were capable of attacking the polysaccharides. If the samples were open to Novozym 342 attack, reducing sugar would be released. The DNS reducing sugar analysis results are provided in Fig. 3.

Fig. 3 indicates that some reducing sugars were also released from the modified extended impregnated materials, which means that although more glucomannan was retained in the materials, the enzymes can still penetrate the wood and attack the polymers. One possible explanation for this result is that approximately 20% of the lignin was lost during the impregnation process, as indicated in table 2. The non-phenolic β -O-4 ethers in the lignin are cleaved under alkaline conditions,^[28] and the covalent bonds between the lignin and polysaccharides may also be broken by the lignin dissolution. This reaction may partly degrade the lignin-carbohydrate networks in the wood, rendering the structure of the wood “looser” and more flexible, thereby allowing the enzymes to penetrate the structure and access the polysaccharides. In addition to the lignin, some hemicelluloses were lost during the extended impregnation, which could also lead to the cleavage of the lignin carbohydrate complex. Because more glucomannan was preserved in the modified extended impregnated samples, the reducing sugar content from the respective samples was slightly lower than that of the extended impregnated sample, except for the AQ-modified material. The percentage of reducing sugar released with the EI-AQ was slightly higher than that with the EI sample and was accompanied by an increased yield, as indicated in Fig. 3. This observation

might be explained by the catalytic cycle, which included both glucomannan and lignin, as presented in Fig. 2. The reduced form of the AQ i.e. AHQ, prevented any undesirable side reactions (e.g., condensation) of the lignin and enhanced delignification.

Combining the stabilisation of glucomannan and the enhanced dissolution of lignin, the compact wood structure may be opened more widely for the enzymes. Thus, the quantity of reducing sugar released from the EI-AQ sample was higher than that from the EI-PS, EI-NaBH₄, and EI samples.

The carbohydrates were stabilised by additives during the extended impregnation as previously described, allowing their isolation during the extraction stage. When no additive was used, the yield of the extracted material was 9% w/w wood, and the gamanase treatment had no noticeable effect on the extraction yield. However, the extended impregnation with sodium borohydride preserved the carbohydrates in the wood. Therefore, 11% w/w wood was extracted from the sodium borohydride-treated sample, which increased to 14% with gamanase treatment. With polysulphide and anthraquinone, the quantity of material extracted was 11–12% w/w wood (Fig. 4), with no significant increase after gamanase treatment. This result may occur because the polysulphide and anthraquinone did not preserve as much polysaccharide as the sodium borohydride.

Table 3 summarises the amount of lignin and anhydro-monosugars isolated from the impregnated wood samples. Because the mannose content of the impregnated wood was only 4% w/w wood, only 13% of the mannose was extracted, which increased to 20% following the gamanase treatment. The sodium borohydride preserved a significant quantity of mannose in the wood; hence, a comparatively larger quantity of 25% of mannose could be extracted, which was increased to 30% with the

gamanase treatment. The material extracted from the non-enzyme-treated wood has a molecular weight ranging up to 20kDa, indicating that the opened wood structure enables the extraction of high-molecular weight materials regardless of any additives. However, the molecular weight of the material extracted from the gamanase-treated wood was not high, and most of the extracted hemicelluloses appear in the oligomeric region with some larger LCCs likely because the gamanase degraded the polysaccharides.

Less xylose was extracted from the gamanase-treated samples regardless of the pretreatment method. The result is unexpected and not easily explained. However, gamanase contains some xylanase activity, and because xylan is fairly soluble in alkali solutions, the xylan in the pretreated material may have been partially dissolved and precipitated, thereby becoming vulnerable to enzymatic degradation. Thus, xylanase-free enzyme products may be of interest for this type of application.

The lignin content of the material extracted from the gamanase-treated wood is also slightly elevated, possibly because the gamanase can reduce the glucomannan, which allowed more free LCCs to be extracted.

CONCLUSIONS

Glucomannan was successfully preserved by the three additives during modified extended impregnation. The yields increased from 76.6% to 80.0% using PS, 81.3% using AQ and 89.6% using NaBH₄. Modified extended impregnation opened the wood to the enzymes, allowing the chemoenzymatic extraction to work. The use of additives during the extended impregnation enhanced the yield of the extracted material with molecular weights as high as 20kDa. The gamanase treatment further improved the

extraction yield, but the polymers were degraded to oligomers or monomers. Therefore, a monocomponent such as endo-mannanase could be a better option than gamanase.

The chemo-enzymatic concept in a biorefinery process was successfully demonstrated with these three additives. But the most efficient additive, NaBH₄, is too expensive for use in a large-scale application. New additives would be continuously researched in the following study.

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Figures

Peeling reaction

Stop reaction

Fig. 1. Peeling reaction of glucomannan and stopping reaction of arabinoxylan

Sodium borohydride reaction

Polysulphide reaction

Anthraquinone reaction

Fig. 2. Mechanisms for stabilization of glucomannan with sodium borohydride, polysulfide and anthraquinone.

Fig. 3. Released reducing sugar analysis and yield comparison. The yield increased from 76.6% to 80.0% (PS), 81.3% (AQ) and 89.6% (NaBH₄) respectively under the same condition, and the reducing sugar analysis data indicated that the enzymes also could penetrate into and attacking the wood.

Fig. 4. Yield of extracted wood residues after enzymatic or non-enzymatic treatment of impregnated wood, in comparison with the yields of the wood after impregnation. Sodium borohydride preserved more carbohydrates than other additive leading to higher extraction yield from EI-NaBH₄. Gamanase treatment improved the extraction to a little extent

Tables

Table 1. Extended impregnation conditions and additives

Extended impregnation	Wood to liquor ratio	1:7
	Alkali charge ([OH ⁻] g/L)	25
	Sulfidity (%)	60
	Maximum temperature (°C)	110
	Impregnated time (min.)	120
Additives charge (w/w%, based on o.d. weight of wood)	NaBH ₄	3
	PS	4
	AQ	0.15

Table 2. Composition of wood impregnated with or without additives presented as % based on original wood

% of wood	Yield	KL.	Ara	Gal	Glu	Xyl	Man	Total monosaccharide content
Raw spruce wood chips	100	28.3	1.57	2.23	46.89	6.88	13.56	71.13
Spruce treated with EI	76.6	22.4	0.88	0.77	44.16	4.85	4.01	54.67
Spruce treated with EI-NaBH ₄	89.5	23.2	1.03	1.37	48.52	5.91	10.17	67.00
Spruce treated with EI-PS	80.0	22.8	0.94	1.12	45.97	5.53	5.76	59.32
Spruce treated with EI-AQ	81.3	22.7	0.84	0.70	45.21	5.68	5.52	57.95

EI: extended impregnation, EI-NaBH₄: extended impregnation with NaBH₄, EI-PS: extended impregnation with elemental sulfur, EI-AQ: extended impregnation with anthraquinone.

Table 3 Amount of lignocelluloses extracted from impregnated wood

% of wood		KL.	Ara	Gal	Glu	Xyl	Man
EI	Non-enzyme treated	5.77	0.07	0.09	2.62	0.20	0.53
	Gamanase treated	6.77	0.08	0.15	2.10	0.20	0.88
EI-NaBH ₄	Non-enzyme treated	4.64	0.26	0.48	2.98	1.20	2.51
	Gamanase treated	5.86	0.11	0.52	4.36	0.50	3.02
EI-PS	Non-enzyme treated	5.76	0.02	0.53	3.64	1.41	1.54
	Gamanase treated	7.27	0.10	0.57	4.08	0.62	1.81
EI-AQ	Non-enzyme treated	5.01	0.11	0.09	2.18	1.32	2.18
	Gamanase treated	6.40	0.10	0.18	1.16	0.58	2.25