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Evaluation of Enzyme Mixtures in Releasing Fermentable Sugars from Pre-pulping Extracts of Mixed Northeast Hardwoods

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Abstract One near-term option to developing a forest product biorefinery is to derive pre-pulping extract from incoming wood chips before the main pulping step. The release of monomer sugars from a xylan-rich extract, creating a fermentable substrate is a prerequisite for utilization of pre-pulping extract for production of ethanol or other value-added products. This study examined the individual and mixture efficiencies of two hemicellulolytic microbial enzymes and two xylanase preparations in catalyzing degradation of green liquor (GL) and hot water (HW) pre-pulping extracts. The effects of four commercial enzyme preparations were determined by assessing yields of xylose + galactose + mannose (xmg) obtained under different reaction conditions. Of the individual enzyme preparations tested, a sample NS 50012 was superior to the other enzyme preparations in releasing xmg under conditions optimized for separate hydrolysis and fermentation and for simultaneous saccharification and fermentation. In comparison to pre-pulping extracts treated with HW, extract treated with GL was found to inhibit the action of all tested enzymes. This inhibition may be related to higher salt and lignin phenol in the GL extract. On both types of extracts, the mixture constituted by NS 50012 and NS 50030 provided the highest yield of hemicellulose conversion at 55 °C and pH 5.5. The generated digestibility thus signified that the synergistic effectiveness in xylan + galactan + mannan (XMG) hydrolysis between NS 50012 (from *Aspergillus aculeatus*) and NS 50030 (from *Aspergillus oryzae*) is the result of an interaction mechanism involving different XMG-degrading enzyme activities in the two enzyme preparations.

Keywords Green liquor · Pre-pulping extraction · Enzymatic hydrolysis · Hemicellulolytic enzyme · Xylanase

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Introduction

A kraft pulp mill-based biorefinery is envisioned as a means of making use of the lower value components in wood that are removed from the pulp and do not contribute to the final fiber product. Pulps may be purified sufficiently to provide raw material for conversion into cellulose derivatives such as rayon, cellulose esters, and cellulose ethers [1]. This requires removal of most of the hemicelluloses by cooking the wood before pulping. Cooking hydrolyzes some of the acetyl groups to produce acetic acid, which in turn autocatalyzes further hydrolysis of the hemicellulose and facilitates its removal during pulping [2, 3]. When this procedure is used prior to kraft pulping, it is known as pre-pulping extraction. Prior to pulping, hemicelluloses and residual lignin may be removed from chemical pulps by extraction with green liquor (GL) or hot water (HW) solutions.

The extraction of hemicellulosic sugars by a near-neutral pH extraction process releases sugars that are mostly oligosaccharides with small amounts of monosaccharides. The near-neutral hemicellulose extraction process involves extracting wood hemicellulose using dilute green liquor prior to conventional kraft pulping [4]. Ancillary unit operations in the process include concentration of the extract, hydrolysis of the extracted carbohydrates using sulfuric acid or enzymes, filtration of the acidified extract to remove lignin, liquid–liquid extraction of acetic acid, liming of the hydrolyzed extract, separation of gypsum which is the product of the liming reaction, fermentation of C5 and C6 sugars, and upgrading of the acetic acid and ethanol products by distillation. Concentration of the polymeric oligosaccharides can be accomplished through evaporation or ultrafiltration. Membrane-based filtration is a less energy intensive method of concentrating the hemicellulosic oligomers, with the added advantage that is does not concentrate salts or acids and has been proposed as a part of ethanol production from extract [5, 6].

Acetyl esters normally present on the hemicellulose are partly cleaved, leaving some esters still attached to the sugars [7]. This complicates a detailed analysis of the sugars present and necessitates a secondary hydrolysis in the analysis of the total carbohydrates present. A second hydrolysis is even more important for the identification of the carbohydrate composition when the hemicellulose is fermented by microorganisms. In green liquor extract, the hemicellulose comes out as a polymeric material and the acetyl esters as acetate. Most arabinoxylans in the extracts are principally composed of a backbone of (1→4)-linked β-D-xylopyranosyl residues where single α-arabinofuranosyl substituents are attached to the C(O)-2, C(O)-3 or to both C(O)-2,3 of the xylose residues [8–11]. The xylan backbone can also be substituted with α-D-glucopyranosyl uronic acid or its 4-*O*-methyl derivative and acetyl groups [12]. In addition, ferulic acid and *p*-coumaric acid may be covalently linked to arabinoxylan via esterification at the C5 position of some of the arabinosyl units [13].

Secondary hydrolysis of arabinoxylans present in extract to fermentable monosaccharides can be accomplished by acid or enzyme catalyzed hydrolysis. The enzymatic process offers some advantages, such as minimization of the formation of decomposition products that inhibit the subsequent microbial fermentation, reduction of acid and base consumption, as well as reduction in gypsum disposal costs, and a more environmentally friendly process [14, 15]. In a previous study, we were able to optimize the xylose yield from acid hydrolysis of the extract with respect to the temperature, residence time, and concentration of the acid catalyst [16]. For the case of enzymatic hydrolysis, optimal conditions depend on all of these factors, as well as substrate composition and enzyme activities. Due to the complexity and heterogeneity of the arabinoxylan structure, a full enzymatic degradation into monosaccharides requires both side-group cleaving and depolymerizing enzyme activities. In hemicellulose degradation, xylan-degrading activities include endo-1, 4-β-xylanases (EC 3.2.1.8) and β-xylosidases (EC

3.2.1.37) which attack the main polymer chain. In addition, other accessory enzymes such as α-L-arabinofuranosidases (EC 3.2.1.55), feruloylesterase (EC 3.1.1.73), α-glucuronidases (EC 3.2.1.139), and perhaps acetylesterases (EC 3.1.1.6) may be required depending on the degree to which the arabinoxylans are entangled with other structures in the substrates [17, 18].

Cellulase enzyme produced by *Humicola insolens* have been widely studied [19, 20], and the production of α-L-arabinofuranosidase, endo-1, 4-β-xylanase, as well as feruloyl esterase activity by *Humicola insolens* is also well documented [21, 22]. *Aspergillus oryzae* and *Aspergillus aculeatus* have also been widely reported as good producers of arabinoxylan-degrading activities [23–26]. In this study, we decided to assess and compare the xylan-, mannan-, galactan (XMG)-degrading efficiency of commercially available hemicellulolytic enzyme and xylanase products produced by these organisms, because of the putatively wide range of relevant activities produced by these different microorganisms.

The purposes of this study were to examine the variations in hydrolysis performance resulting from different enzyme types, GL versus HW extractions, and synergies achievable through use of blended enzyme mixtures. We also examined the difference in hydrolysis performance resulting from pH and temperature effects at conditions representative of simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) operating conditions.

Materials and Methods

Raw Material

Chips of mixed hardwood were supplied by the Red Shield (currently Old Town Fuel and Fiber) kraft pulp mill in Old Town, ME, USA. The chips were screened to an average 7/8–5/8 in (22.6~16.0 mm) using a mechanically vibrated horizontal screen. The screened wood chips were used directly in the extraction studies. Some of the screened chips were ground to an average size of 30~40 mesh (0.595~0.420 mm) using a laboratory knife mill. The milled wood chips were used for determination of total solids/moisture and carbohydrate content in biomass. The sugar composition of un-extracted wood chips was 42.1% glucan, 23.1% XMG, and 1.8% arabinan [16, 27]. Note that XMG (capitalized) represents the sum total of the three oligomeric sugars xylan, mannan, and galactan while xmg (small letters) represents the sum of the corresponding hydrolyzed sugars xylose, mannose, and galactose.

Green Liquor

Green liquor (GL) is the partially recovered form of kraft-pulping liquor. It is obtained after combustion of the black liquor in the recovery boiler by dissolving the smelt from the recovery boiler (Na_2S, Na_2CO_3, and any impurities) in water. In this study, pure chemicals (Na_2S: sodium sulfide hydrate, 65% extra pure; Na_2CO_3: sodium carbonate monohydrate 99.5% extra pure; Na_2SO_4: sodium sulfate anhydrous, 99.2% extra pure; NaOH: sodium hydroxide pellets, 98.5% extra pure; Fisher, Pittsburgh, PA, USA) were used. The composition of the green liquor used in the study is listed in Table 1.

Green Liquor and Hot Water Extraction

A hemicellulose extraction was performed using a 20 L rocking digester at the University of Maine Process Development Center (Fig. 1). A stainless steel basket in the digester was

Table 1 Chemical composition of green liquor used in extraction process.

Chemicals	Value as Na_2O
Total titrated alkaline (TTA)	3% on wood as Na_2O
Sodium hydroxide (NaOH)	9.0 g/L as Na_2O
Sodium sulfide (Na_2S)	29.1 g/L as Na_2O
Sodium carbonate (Na_2CO_3)	70.0 g/L as Na_2O
Sodium sulfate (Na_2SO_4)	0.8 g/L as Na_2O
TTA	108.9 g/L as Na_2O

loaded with 2 kg of air-dried chips of average dimension-16 mm×22.6 mm×4 mm. The residual moisture in the air-dried wood was accounted for in the determination of the amount of GL to be added, which gave a final ratio of 4 L liquor per kilogram oven dry wood: 0.55 L of GL was blended with 6.35 L of water and added to 3.1 kg of 36.1% moisture wood. In the case of extraction with HW, the same liquor-to-wood ratio was used without any GL charge. The rocking digester was oscillated from the vertical, upright position to 90° in two lateral directions. This system was slowly rocked at 2 rpm and heated up to160 °C for 110 min, and then cooled yielding an H-factor of 800. The H factor is an

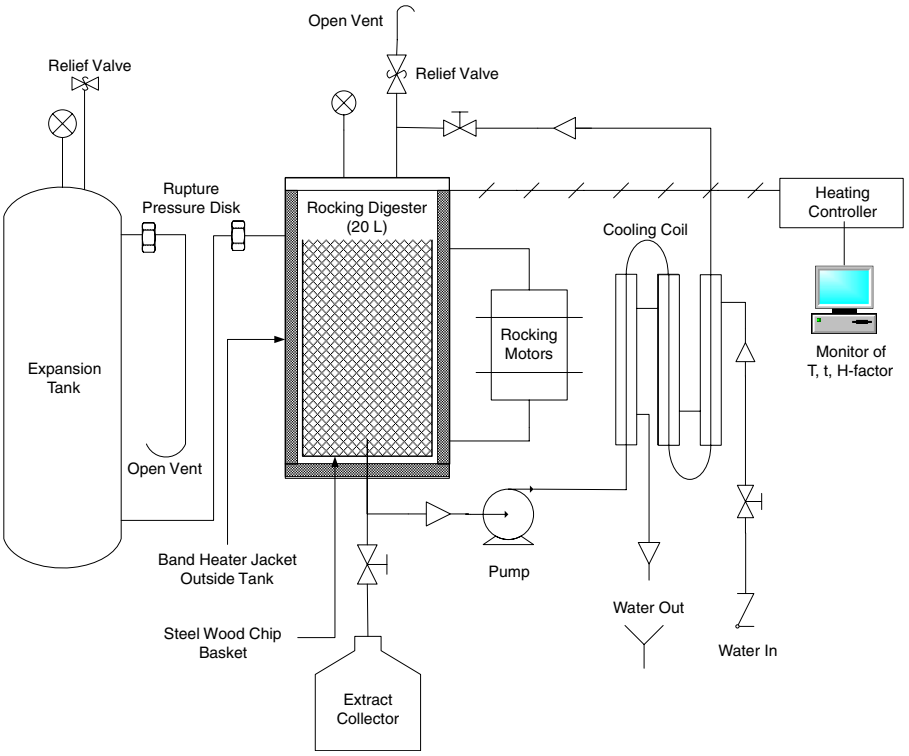

Fig. 1 Schematic diagram of the rocking digester apparatus used to prepare extract samples

integration of temperature-dependent reaction rate over time, as shown in Eq. 1 [28]. The equation is

$$H = \int_{t=0}^{t=t} e^{[43.181-(16{,}113/T)]}\,\mathrm{dt} \tag{1}$$

in which t is given in minutes and T in degrees Kelvin. The free-draining extract was collected from the digester, then analyzed and prepared for subsequent enzyme hydrolysis experiments. The total volume of the drained extract ranged from 5 L to 6 L after one reaction (H=800).

Membrane Filtration

In this study, tangential flow filtration was performed for concentrating the XMG using a Millipore Pellicon two system with two membranes. First a polyvinylidene fluoride membrane with pore size of 0.45 μ was used to precondition the extract and remove relatively large particles. This was followed by a polyethersulfone membrane having a nominal molecular weight cutoff of 1,000 Da that was used to concentrate the oligomers. The membranes each had a surface area of 0.1 m^2. The system was operated at a feed pressure of 12 psi and retentate pressure of 8 psi. De-ionized water was used to purge the system prior to the introduction of the hemicellulose extract. Filtration started with 500 mL HW extract and 500 mL GL extract. Approximately 150 mL were purged through the system before the retentate was allowed to recycle back to the feed tank. Permeate was collected from the top of the membrane cassette. After 30 and 120 min for hot water and green liquor, respectively, 320 mL of permeate and 125 mL of retentate were collected. The retentate was prepared for subsequent enzyme hydrolysis experiments with some samples of the retentate taken and hydrolyzed with 4% (*w*/*v*) sulfuric acid to determine the sugar concentrations by high performance liquid chromatography (HPLC).

Commercial Enzymes

The Novozymes preparations NS 50012, NS22002, NS50014, and NS50030 were kindly supplied by Novozymes, North America Inc. The enzymes were contained in a Novozymes Biomass Kit intended for conversion of lignocellulosic materials. The main characteristics of each enzyme preparation are described in Table 2. The sample NS50012 is a multi-enzyme complex containing a wide range of carbohydrases, including arabinase, β-glucanase, cellulase, hemicellulase, pectinase, and xylanase. The NS22002 contains a mixture of β-glucanase and xylanase enzyme activities. β-glucanase and xylanase are the two main enzyme activities in the enzyme preparation, but the product also contains several other side activities, including cellulase, hemicellulase, and pentosanase. The enzyme samples of NS50014 and NS50030 are endo-xylanase with a high specificity towards soluble pentosans.

Enzyme Hydrolysis Experiments

Enzymatic hydrolysis was performed on the GL- and HW-treated extract using the National Renewable Energy Laboratory (NREL) Standard Procedure # 009 [29]. Enzyme experiments were conducted with commercially available enzymes described in Table 2. The individual

Table 2 Summary of enzyme preparation characteristics.

Enzyme name	Main activities	Source	Optimal pH	Optimal temperature (°C)	Density (g/mL)
NS50012	Enzymec omplex I (100 FBG/g)[a]	*Aspergillus aculeatus*	4.5–6.0	25–55	1.2
NS22002	Enzyme complex II (45 FBG/g)	*Humicola insolens*	5.0–6.5	40–60	1.2
NS50030	Xylanase I (500 FXU/g)[b]	*Aspergillus oryzae*	4.5–6.0	35–55	1.1
NS50014	Xylanase II (650 FXU/mL)	*Aspergillus oryzae*	5.0–8.0	45–70	1.2
Tested mixtures	Enzymes included, equal ratio of nominal units		Assumed optimal pH	Assumed optimal temperature (°C)	
ME1	NS50012 + NS50030		5.5	55	
ME2	NS50012 + NS50014		5.5	55	
ME3	NS22002 + NS50014		5.5	55	
ME4	NS22002 + NS50030		5.5	55	
ME5	NS50030+NS50014		5.5	55	

Enzyme samples and information were supplied by Novozymes North America Inc (Franklinton, NC, USA).

[a] *FBG* Fungal β-glucannase unit: 1-β-glucanase unit (BG) is the amount of enzyme, which under standard conditions liberates glucose or other reducing carbohydrates with a reduction power corresponding to 1 μmol glucose per minute under the optimal conditions listed.

[b] *FXU* Farvet Xylan Unit is measured relative to a Novozymes FXU enzyme standard. The FXU activities are determined relative to a reference enzyme standard. The activity of the reference standard, *Humicola insolens,* xylanase batch no. 17-1194, is defined to have an enzymatic activity of 3,550 FXU/g at pH 6.0 and 50 °C in 30 min reaction time of colour release from remazol-xylan substrate (from Novozymes Biomass Kit for conversion of lignocellulosic materials).

effects of two different operating conditions were evaluated for each enzyme preparation. The first operating condition was at pH 7.0 and temperature 37 °C. This was selected to be compatible with the optimal conditions for a fermentation using the cofermenting *Escherichia coli* KO11 in SSF mode [30]. The second conditions selected were based on the reported optimal ranges for the enzymes of pH (4.5–6.0) and temperature (35–70 °C). The mid points of these ranges were selected, i.e., pH 5.5 and temperature 55 °C. Enzyme dosage of the individual enzymes and enzyme mixtures was calculated by suggested NREL Standard Procedure # 008 [29]. The equation used is

$$x\,g\,\mathrm{XMG} \times \frac{y\,\mathrm{FBG\,(or\,FXU)}}{g\,\mathrm{XMG}} \times \frac{1}{z\,\mathrm{FBG\,(or\,FXU)/mL}} = E\ \mathrm{mL} \tag{2}$$

where, x=g of XMG in the extract, y=loading enzyme activity, z=activity of enzyme solution, and E=loading enzyme amount. The enzyme loading was adjusted to attain a range of activity from 50 to 350 FBG (fungal β-glucannase) or Farvet Xylan Unit (FXU)/g XMG in the flasks. To evaluate synergistic effects, all of the paired enzymes were tested by preparing mixtures that each contained nominal activity (FBG or FXU) ratios of 50:50, 75:75, and 350:350 between the combined enzyme mixtures. All hydrolysis reactions were run at the above two pH/temperature conditions for 96 h. Experiments were conducted in

125 mL Erlenmeyer flasks with a working volume of 20 mL containing 80 μL tetracycline and 60 μL cycloheximide on a New Brunswick Scientific incubated shaker. pH of these samples were adjusted with calcium hydroxide. Samples were withdrawn after 24, 48, 72, and 96 h, heat denatured (boiling water for 10 min) to inactivate and precipitate the enzyme, and then processed for HPLC sugar and acetic acid analysis. All experiments were performed in duplicate. The enzymatic digestibility was calculated as the concentration of the hydrolyzed xmg divided by the initial xmg content in the extract and expressed as percentage. XMG digestibility was calculated as follows Eq. 3:

$$\%\text{XMG digestion} = \frac{[\text{xmg}]_f - [\text{xmg}]_o}{[\text{xmg}]_t - [\text{xmg}]_o} \times 100 \tag{3}$$

Where the lower case xmg represent the combined concentration of xylose, mannose, and galactose monomers in solution, $[\text{xmg}]_f$=xmg concentration at the end of the enzyme hydrolysis (g/L), $[\text{xmg}]_o$=xmg concentration at the beginning of the enzyme hydrolysis (g/L), $[xmg]_t$=theoretical maximum concentration of xmg contained in the extract at the beginning of the enzyme hydrolysis (g/L).

High Performance Liquid Chromatography

The sugar composition of reaction products was quantitatively analyzed by HPLC. The Shimadzu model (LC-10AT Liquid Chromatogram, Shimadzu Corp., Kyoto, Japan) HPLC used for carbohydrate measurement had a Bio-Rad Aminex HPX-87H column (300 mm× 7.8 mm, Bio-Rad Laboratories Inc., Hercules, CA, USA) and Cation H micro-guard cartridge (30 mm×4.6 mm, Bio-Rad Laboratories Inc., Hercules, CA, USA). The column was maintained at 60 °C, with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 mL/min. All of the sugar and acid peaks were detected by a refractive index detector and ultraviolet absorption (280 nm) and were identified and quantified by comparison to retention times of authentic sugar and acid standards. The Bio-Rad Aminex HPX-87H analytical column allows the concurrent analysis of liquid sample for the presences of acetic and lactic acids.

Results and Discussion

Substrate extracted by GL and HW

The yield of hemicellulose extracted is relatively small in the pre-pulping extraction process. This is especially true when compared to hemicellulose extraction from lignocellulosic biomass by dilute acid pretreatment. The low extraction yield is maintained because of concerns over loss of pulp yield and quality. The concentrations of xmg released from mixed hardwood chips following HW and GL extraction are shown in Table 3. Under the extraction conditions used, approximately 5.0% for HW and 3.1% for GL of the wood mass is extracted as carbohydrates. The initial aqueous extracts had a solids content of 3.7% (w/w), a pH=3.5 for HW extract and 3.3% (w/w), a pH=5.2 for GL extract. Extraction using only HW generates an acidic extract due to the un-buffered release of acetyl groups from wood, which may then result in degradation of hemicellulose fiber by acid hydrolysis. The concentration of GL used in this study (3% Na_2O equivalent on wood) was chosen because it had been previously demonstrated to preserve the quantity and quality of the

Kraft pulp that could be produced with the wood solids remaining after extraction [4]. Higher concentrations of GL have been shown to result in lower carbohydrate concentrations in the liquor, while lower GL concentrations tend to yield more sugars but degraded pulp quality and quantity [31].

Each of these extracts was hydrolyzed by NREL procedure (# 002) using sulfuric acid and the resulting theoretical maximum yield is presented in Table 3. The raw extracts are dilute after extraction, so an ultra filtration was used to increase oligosaccharide concentration prior to hydrolysis. From the raw extraction liquor containing 11.11 and 7.04 g/L total carbohydrates for HW and GL extract, respectively (Table 3), the concentrated hemicellulose extracts used in this study contained 1.54 g/L glucan, 26.71 g/L XMG and 1.18 g/L arabinan for HW extract and 0.61 g/L glucan, 7.98 g/L XMG and 1.62 g/L arabinan for GL extract, which make up total carbohydrate contents of 29.52 and 10.97 g/L, respectively (Table 4). The concentration of acetic acid was increased for GL extract from 6.80 to 9.32 g/L through this filtration, which is potentially high enough to have significant inhibitory effects on the ethanol fermentation. It is thought that this increase in acetic acid results from acetyl groups still bound to the oligosaccharides, which are retained by the filtration.

Evaluation of Individual Enzyme Effects on Sugar Yield

The original HW extraction liquor containing 9.94 g/L XMG (Table 3) was used to evaluate individual enzyme effects under different hydrolysis conditions of pH, temperature, and enzyme dosage. Specifically, the pH and temperature range were selected for SSF and SHF conditions, the latter of which would include hydrolysis conditions optimized to enzyme characteristics.

As expected, the digestibility levels of XMG varied significantly in response to the different enzyme samples and dosage in the selected pH and temperature ranges (Fig. 2). Hydrolysis with enzyme complex I (NS 50012) generally resulted in superior release of xmg as compared to the other enzyme treatments at both conditions of pH and temperature. Enzyme complex II (NS 22002) hydrolysis also efficiently catalyzed the release of xmg, but gave slightly lower xmg yields than that of NS 50012. The results from the SSF conditions of 37 °C and pH=7.0 and SHF conditions of 55 °C and pH=5.5 are plotted in Fig. 2 as a

Table 3 Composition of raw HW and GL extracts after the extraction process.

	Reagent	Glucan [g/L]	XMG [g/L][a]	Arabinan [g/L]	Lactic acid [g/L]	Formic acid [g/L]	Acetic acid [g/L]	Total sugar [g/L]
Theoretical maximum yield[b]	HW	0.62	9.94	0.55	0.04	0.05	3.24	11.11
		±0.01	±0.16	±0.01	±0.02	±0.00	±0.13	±0.18
	GL	0.41	5.92	0.71	0.62	0.25	6.80	7.04
		±0.04	±0.50	±0.30	±0.03	±0.01	±0.03	±0.84
Raw extracts: blind control	HW	0.15	1.62	0.42	0.04	0.04	1.47	2.19
		±0.00	±0.03	±0.01	±0.00	±0.00	±0.01	±0.04
	GL	0.12	0.14	0.22	0.55	0.13	2.35	0.48
		±0.02	±0.05	±0.03	±0.00	±0.01	±0.03	±0.10

HW (hot water), GL (green liquor).

[a] XMG (capitalized) represents the sum total of the oligomeric sugars xylan, mannan, and galactan.

[b] Theoretical maximum yield was determined by acid hydrolysis (NREL procedure # 002).

Table 4 Composition of filter concentrated HW and GL extract after tangential flow filtration (TFF).

	Reagent	Glucan [g/L]	XMG [g/L][a]	Arabinan [g/L]	Lactic acid [g/L]	Formic acid [g/L]	Acetic acid [g/L]	Total sugar [g/L]
Theoretical maximum yield[b]	HW	1.54	26.71	1.28	0.08	0.12	4.96	29.52
		±0.01	±0.10	±0.03	±0.03	±0.00	±0.01	±0.14
	GL	0.61	7.98	1.62	0.85	0.36	9.32	10.97
		±0.03	±0.30	±0.02	±0.00	±0.04	±0.02	±0.35
Concentrated extracts: blind control	HW	0.39	3.06	1.17	0.06	0.10	2.25	4.72
		±0.00	±0.19	±0.03	±0.01	±0.01	±0.09	±0.22
	GL	0.29	0.19	1.24	0.76	0.31	8.07	1.72
		±0.07	±0.00	±0.03	±0.02	±0.004	±0.02	±0.10

HW (hot water), GL (green liquor).

[a] XMG (capitalized) represents the sum total of the oligomeric sugars xylan, mannan, and galactan.

[b] Theoretical maximum yield was determined by acid hydrolysis (NREL procedure # 002).

function of enzyme activity. The experimental maximum yield for xmg after 72 h production was found to be 49.7% with 50 FBG loading of NS 50012 and 56.4% with 350 FBG loading of NS 50012 (Fig. 2a). Figure 2b shows results for XMG conversion of raw HW extract after 72 h with a mixture of enzymes. Mixture ME1 (NS 50012 and NS 50030) provided the highest yield of xmg, whereas with the pure xylanases, mixture ME5 (NS 50030 and NS 50014) did not significantly affect the xmg liberation at either hydrolysis condition (Fig. 2b).

Figure 2 summarizes the xmg sugar yields with constant enzyme loading under the two different temperature and pH conditions. When comparing the sugar yield obtained with the individual enzyme preparations, the NS 50012 and NS 22002 were the best at catalyzing an optimal xmg release at both SHF and SSF conditions. Figure 2b illustrates that the maximum percent sugar yield occurred at the temperature of 55 °C and at pH 5.5.

Effect of Mixed Enzyme Preparations on XMG Hydrolysis

As expected, it was found that higher enzyme dosage, higher temperature (55 °C), and more prolonged reaction times resulted in better xmg yields obtained with NS 50012 and NS 22002 alone. XMG hydrolysis was comparatively lower after NS 50030 and NS 50014 treatments, which was likely due to the lack of effective combinations of hemicellulase enzymes and endo-1,4-β-xylanase activities in these single preparations. To further investigate the benefits of mixed enzyme applications, tests were carried out to determine the optimal combinations of the four enzyme preparations in the hope of reducing the total dosage of enzyme for xmg release during hydrolysis.

In Tables 5 and 6, the XMG digestibility from the filter concentrated HW and GL extracts are compared after 72 h with different combinations of enzyme mixtures under various operating conditions. It was found that the efficiency of hydrolysis with 50:50 (SHF conditions) and 350:350 (SSF conditions) nominal unit mixtures of ME1 (NS50012 + NS50030) resulted in the highest synergistic release of xmg under all of the tested reaction conditions. The different combinations of paired enzymes corresponded to a range of enzyme dosages ranging from 0.1% to 2.5% (*v*/*v*) for 50:50 nominal unit combinations

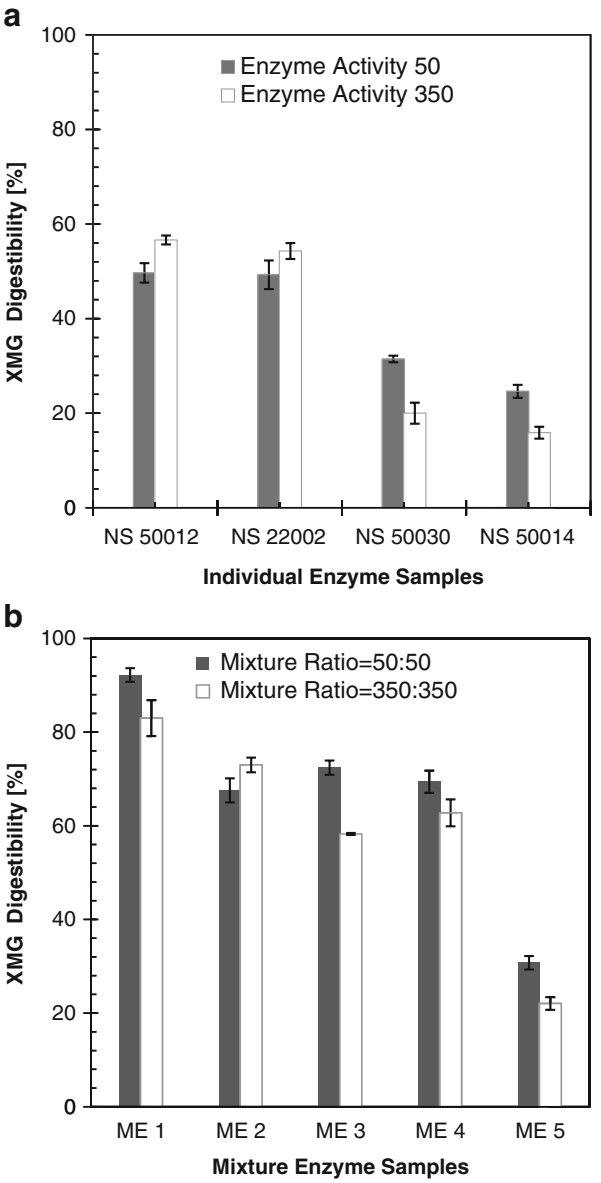

Fig. 2 Enzymatic hydrolysis of raw HW extracts by individual enzymes (**a**) and mixtures of enzymes (**b**). *Dark bars* SSF hydrolysis conditions (T=37 °C, pH =7.0, RPM =160, t=72 h) with high enzyme loading (350 FBG or FXU); *open bars* SHF hydrolysis conditions (T=55 °C, pH =5.5, RPM =160, and t=72 h) with lower enzyme loading (50 FBG or FXU)

(Table 5), and a dosage range of 0.4% to 17.7% (*v*/*v*) for 350:350 nominal unit combinations (Table 6). The mixture ME1 (50:50) catalyzed release of 21.4 and 4.4 g/L of xmg and 4.2 g/L and 9.2 g/L of acetic acid from filter concentrated HW and GL extracts, respectively. Both substrates were treated at 55 °C and pH 5.5. As can be seen in Table 6, the maximum yield on HW extract with ME1 at a 350:350 blend at 37 °C, pH 7.0, and 72 h was 20 g/L of xmg and 4.0 g/L of acetic acid. The yield under these conditions on GL extract was 3.2 g/L of xmg and 8.2 g/L of acetic acid. The binary mixture ME1 of 1.0 vol.% NS50012 and 0.2 vol.% NS50030 ([*v*/*v*] E/S) seems to be the most suitable for the XMG hydrolysis of filter concentrated HW extract.

Table 5 Yield range of xmg and acetic acid released from filter-concentrated HW and GL extract during mixed enzyme hydrolysis.

Enzyme mixture	Activity mixture [FBG and FXU/g $_{XMG}$]	Enzyme dosage [%, *v*/*v* (=E/S)][a]	xmg [g/L]	Acetic acid [g/L]	Digestibility at 72 h [%]
			23.7±0.10	5.0±0.01	
ME1	50 FBG+50 FXU	1.0+0.2	21.4±0.46	4.2±0.11	90.2±1.86
ME2	50 FBG+50 FXU	1.0+0.2	18.5±0.60	3.9±0.15	77.9±2.44
ME3	50 FBG+50 FXU	2.5+0.2	17.5±0.30	4.7±0.00	74.0±1.22
ME4	50 FBG+50 FXU	2.5+0.2	17.6±0.60	4.6±0.12	74.3±2.33
ME5	50 FXU+50 FXU	0.2+0.2	6.8±0.40	3.3±0.00	28.9±1.64
Substrate: filter-concentrated HW extract					
Enzyme mixture	Activity mixture [FBG and FXU/g $_{XMG}$]	Enzyme dosage [%, *v*/*v* (=E/S)][a]	xmg [g/L]	Acetic acid [g/L]	Digestibility at 72 h [%]
			7.8±0.30	9.3±0.01	
ME1	50 FBG+50 FXU	0.3+0.1	4.4±0.03	9.2±0.13	56.1±0.57
ME2	50 FBG+50 FXU	0.3+0.1	3.7±0.03	9.3±0.15	47.3±0.56
ME3	50 FBG+50 FXU	0.8+0.1	2.3±0.07	9.1±0.00	29.5±1.31
ME4	50 FBG+50 FXU	0.8+0.1	1.8±0.10	9.3±0.00	23.7±1.12
ME5	50 FXU+50 FXU	0.1+0.1	0.9±0.01	9.4±0.10	10.9±0.28
Substrate: filter-concentrated GL extract					

[a] *[E/S]* total amount of loading enzyme/working volume of extract, initial pH=3.47 (raw HW extract), 5.05 (raw GL extract): pH was adjusted to 5.5 with calcium hydroxide. Enzymatic hydrolysis condition: 55 °C, 160 RPM, for 72 h. pH and temperature levels appropriate for SHF.

Table 6 Yield range of xmg and acetic acid released from filter-concentrated HW and GL extract during mixed enzyme hydrolysis.

Enzyme mixture	Activity mixture [FBG and FXU/g $_{XMG}$]	Enzyme dosage [%, *v*/*v* (=E/S)][a]	xmg [g/L]	Acetic acid [g/L]	Digestibility at 72 h [%]
Substrate: filter-concentrated HW extract					
			23.7±0.10	5.0±0.01	
ME1	350 FBG+350 FXU	7.3+1.6	20.0±0.45	4.0±0.00	84.0±1.83
ME2	350 FBG+350 FXU	7.3+1.4	16.8±0.63	4.0±0.12	70.9±2.57
ME3	350 FBG+350 FXU	17.7+1.6	15. 3±0.30	3.8±0.10	64.5±1.21
ME4	350 FBG+350 FXU	17.7+1.4	14.4±0.22	3.7±0.00	60.7±2.44
ME5	350 FXU+350 FXU	1.6+1.4	5. 5±0.10	2.1±0.03	23.0±0.37
Substrate: filter-concentrated GL extract					
			7.8±0.30	9.3±0.01	
ME1	350 FBG+350 FXU	2.3+0.5	3.2±0.05	8.2±0.12	41.1±0.58
ME2	350 FBG+350 FXU	2.3+0.4	2.4±0.13	8.1±0.00	30.7±1.83
ME3	350 FBG+350 FXU	5.7+0.5	1.7±0.01	7.8±0.11	22.1±0.17
ME4	350 FBG+350 FXU	5.7+0.4	1.4±0.20	7.9±0.00	18.1±0.22
ME5	350 FXU+350 FXU	0.5+0.4	0.5±0.02	7.8±0.22	6.4±0.34

[a] *[E/S]* total amount of loading enzyme/working volume of extract, initial pH =3.47 (HW extract), 5.05 (GL extract): pH was adjusted to 7.0 with calcium hydroxide. Enzymatic hydrolysis conditions: 37 °C, 160 RPM, for 72 h. The pH level and temperature was representative for SSF with *E. Coli* KO11.

Enzyme Digestibility of GL vs. HW Extracts

Mixture ME1 at pH 5.5 and 55 °C after 72 h liberated 90.2% of the XMG from filter concentrated HW extract, while the GL extract, hydrolyzed at the same conditions gave only 56% total XMG conversion. Under SSF conditions, the ME1 mixture of 350:350 nominal units gave 84.0% conversion of XMG in the filter concentrated HW extract compared to 41.1% for GL extract at 37 °C and pH 7.0 after a reaction time of 72 h. Meanwhile, the digestibility of the filter concentrated extracts is below 28.9% with mixture ME5 (NS50030 and NS 50014) under the same conditions. The results obtained thus show that a synergistic interaction was found between the XMG hydrolyzing side activities in proper combinations of hemicellulosic and endo-1, 4-β-xylanase activities.

Effect of Enzyme Loading and Salt on XMG Conversion

For production of bioethanol, biomass pretreatment and the cost of enzymes are critical targets for process and cost improvements [32–34]. Thus, it is important to minimize the addition of enzymes by using optimal hydrolysis conditions procuring easily fermentable carbohydrates. Figure 3 demonstrates the effect of increasing loadings of mixed enzymes on the xmg yield. With mixture ME1 (50:50) at 55 °C and pH 5.5, the xmg production resulted in 56% and 90% of the xmg released from GL and HW extracts at 72 h, respectively.

Increasing the enzyme loading ratio from 50:50 to 75:75, the maximum xmg yield of 98% for HW was achieved in 72 h. A further lift of the enzyme loading increased the sugar yield only slightly, as found by Wyman et al. [35].

In enzyme hydrolysis, there are multiple parameters that influence hydrolysis of homogeneous extracts (e.g., acetic acid, salts, and lignin phenol content) and result in complex reaction kinetics. The lower digestibility of extract produced by GL compared to HW extract might be explained by the higher content of lignin-derived phenolics, acetic acid, or salt of the GL treated mixed hardwood. Among the inhibitors, the effect of acetic acid and GL salt were evaluated by adding these compounds into a sample of HW extract. The enzyme hydrolysis of HW extract was done with enzyme mixture ME1 in the presence of different acetate levels (added as sodium acetate up to 10 g/L) at pH 5.5 to simulate the expected level of acetate inhibition. As shown in Fig. 4a, it was found that acetic acid did not significantly affect the ultimate concentration of xmg obtained. Additional baseline performance testing was done to investigate the inhibitory effects of salts derived from GL. The same molar concentration of each compound shown in Table 1 was chosen to compare their effects on the hydrolysis. After addition of sodium salts to the HW extract, no change was detected for XMG conversion after 96 h at 0 and 10 g/L sodium, whereas the XMG digestibility was dramatically decreased at the sodium concentration of 100 g/L (Fig. 4b). Thus, while a great increase in sodium levels does result in inhibition, sodium levels representative of levels usually found in GL extracts did not appear to be the main cause of reduced hydrolysis activity.

Conclusion

Under conditions optimized to preserve yield and quality of Kraft pulp, the mass of wood extracted into the GL and HW extracts was approximately 11% of the debarked wood mass, which results in a dilute solution of oligomeric hemicellulose sugars. Once hydrolyzed, the HW and GL extraction liquors used in this study contained 11.11 and 7.04 g/L total

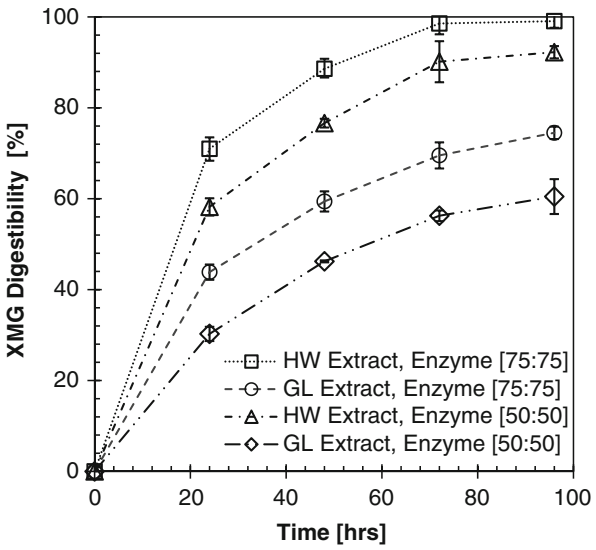

Fig. 3 Enzymatic hydrolysis of filter-concentrated HW and GL extracts by enzyme mixture ME1 at T=55 °C, pH =5.5, and RPM =160

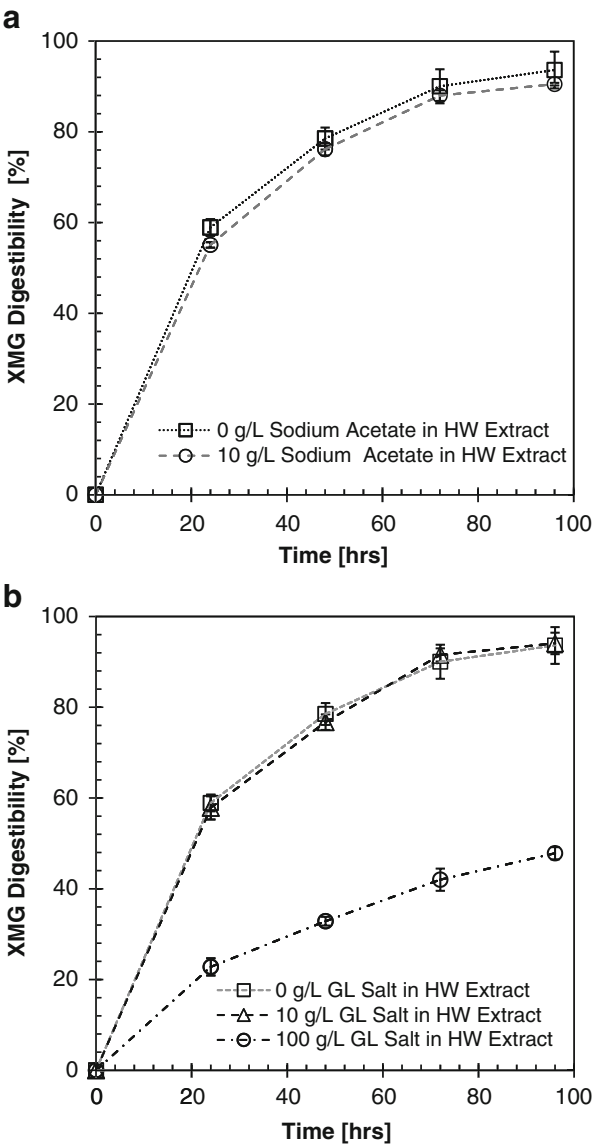

Fig. 4 Effect of acetate (**a**) and green liquor salt concentration (**b**) on the xmg production from HW extract during enzymatic hydrolysis by the enzyme mixture ME1 with activities 50:50 at T=55 °C, pH =5.5, and RPM =160

carbohydrates monosaccharides, respectively. To increase the concentration of sugars, the extract could be concentrated through micro-filtration prior to hydrolysis. Hydrolysis with commercially available enzyme preparations and mixtures of these enzymes was investigated as a method to convert the extracted oligomers to fermentable monomeric sugars. The maximum xmg concentration on concentrated HW extract with a 75:75 nominal units blend of NS50012 and NS50030 at 55 °C and pH 5.5 after a reaction time of 72 h was 23.3 g/L of xmg, representing 98.6% of the maximum possible yield, while the GL extract, hydrolyzed under the same conditions gave only 69.5% total xmg yield. The results of these experiments demonstrated that despite differences in pH optima for the different enzyme preparations, hydrolysis improvements were achieved at average reaction conditions of pH

and temperature with respect to the individual enzyme activity optima. Hydrolysis under conditions suitable for SSF processing (37 °C, pH 7.0) progressed much more slowly and an increase in enzyme concentration on the order of sevenfold were required to achieve similar but often still inferior results. Because the extracts have low concentrations of sugars, the reduction of feedback inhibition on hydrolysis, a principle advantage of SSF processing, is also expected to be minor. It appears that in separate hydrolysis and fermentation, enzymatic hydrolysis presents a viable option for conversion of hot water wood extract oligomers to monomeric sugars, while green liquor extracts appear to be less amenable to complete enzymatic hydrolysis. The cost effectiveness of the enzymes vis a vis acid hydrolysis remains to be determined.

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