PREPARATION OF PRETREATED BIOMASS

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TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

Ctec2	CellicCtec2
Htec2	CellicHtec2
DM	Dry matter
Temp	Temperature

EXECUTIVE SUMMARY

Using an innovative wet explosion pretreatment process, we have optimized the process and prepared pretreated samples from feedstock supplied by the partners in accordance with their specific needs. The specific operational conditions for the different biomass materials were determined with an end focus on FS-10.

The group has further evaluate the samples produced for initial release of C5 and enzymatically derived C6 sugars using commercial enzyme products as well as the level of inhibitory compounds such as HMF, furfural and acetic acids.

The results show that a high quality, fermentable sugar stream, can be produced from the hetero-xylan from pretreatment only; it is also shown that a high yield of glucose from cellulose during enzymatic hydrolysis results in a high quality fermentable C6 fraction and a high quality lignin fiber as a valuable by product.

INTRODUCTION

The major constituent of plant biomass is lignocellulose. Lignocellulose consists mainly of polysaccharides such as cellulose and hemicelluloses that together, with the phenolic lignin polymer, form a complex and rigid structure that is highly resistant towards biological and chemical degradation. Cellulose is typically the most abundant component. It is a homogenous linear polymer of C6 beta-D-glucosyl units linked by 1, 4-beta-D-glucosidic bonds with the degree of polymerization of native cellulose being in the range of 7,000-15,000. The cellulose chains are assembled in larger rigid units held together by both intrachain and interchain hydrogen bonds and weak van der Wall's forces. Through parallel orientation, the chains form a highly ordered crystalline domain, but are interspersed with amorphous regions of more disordered structure.

The second most abundant component, hemicellulose or hetero-xylan, is a highly branched, polymer of pentoses, hexoses, and sugar acids. The degree of heterogeneity, the distribution of the different sugars, as well as the extent of branching is plant dependent. Xylan, which has a backbone of D-xylose linked by 1,4-beta bonds, is the most abundant hemicellulose. The C5 sugars consist primarily of heterosaccharide branched polymers sometimes referred to as glucuronoxylan or O-acetyl-4-methylglucurono-β-D-xylan, but more often are referred to simply as xylan.

Pretreatment is crucial as a first step for breaking this biomass rigid structure; it aims at increasing the accessibility of the polymers for the following enzyme hydrolysis. Several different pretreatment strategies have been suggested over the last years, ranging from extreme temperatures, pressures, and acid/base conditions including (but not excluding others) alkali-, acid-, or organosolvent pretreatement, steam-, ammonia fiber- or CO_2 explosion, and wet-oxidation to the milder biological approaches. The characteristics of the lignocellulosic biomass after pretreatment with regard to cellulose accessibility, degree of polymerization, hemicellulose content, lignin content, and other compounds potential interfering enzymatic hydrolysis, will vary to a great extent depending on the plant material and the pretreatment applied.

A pretreatment process combining wet oxidation and steam explosion (WEx) was developed and patented in 2003. This process has been in pilot testing since 2006 with great success and is now being operating in demonstration scale for production of cellulosic ethanol. In 2010 CleanVantage, LLC started their work on optimizing this technology together with a large US based industrial equipment developer. This work has now resulted in an optimal process and equipment which is ready for implementation and use in industrial scale and which is protected by several patent applications.

The major advantage of the wet explosion process (WEx) compared to any other process on the market is that no external chemicals are added except water and oxygen or air. Furthermore, the process can generally be operated at lower process temperatures than seen for other pretreatments, thus the severity of the pretreatment is low which has an immediate effect on the amount of inhibitors present (low amount of HMF and furfural) which again affects the outcome of both enzymatic hydrolysis and fermentations. Under high temperature and pressure, oxygen will specifically attack the lignin molecules, which will be partly ignited under the process. A fraction of the lignin in the material will therefore be oxidized into lower molecular lignin compounds.

An enzyme cocktail of different glycoside hydrolases is needed for complete breakdown of the long chain sugars after pretreatment to yield monomeric sugars. Especially efficient cellulose degradation is important for producing cheap sugars. The three main categories of players in cellulose hydrolysis are cellobiohydrolases (EC 3.2.1.91), endo-1,4-beta-glucanases (EC 3.2.1.4), and beta-glucosidases (EC 3.2.1.21). Cellulose polymers are through sequential and cooperative actions of these enzymes degraded to glucose, Figure PPB-1. The general consensus is that cellobiohydrolases hydrolyze the cellulose polymer from the ends, releasing cellobiose as product in a progressive fashion. Endo-glucanases randomly hydrolyze the internal 1,4-beta-linkages in primarily the amorphous regions of cellulose, rapidly decreasing the degree of polymerization and creating more free ends for the cellobiohydrolases. Finally, beta-glucosidases hydrolyze the cellobiose and in some cases the cello-oligosaccharides to glucose. Cellobiohydrolases and endoglucanases are often inhibited by cellobiose, making beta-glucosidases very important in terms of avoiding decreased hydrolysis rates of cellulose over time due to cellobiose accumulation. Maintaining high hydrolysis rates ultimately depend on the beta-glucosidases, making them bottleneck enzymes for efficient hydrolysis of cellulose.

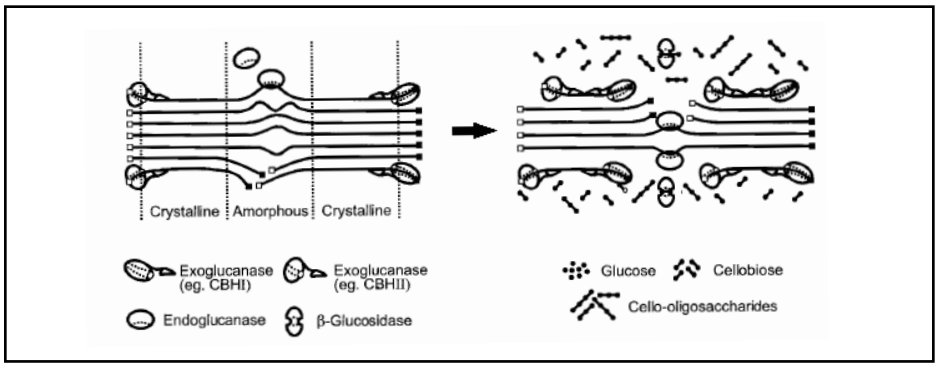

Figure PPB-1. Cellulose hydrolysis: Sequential and cooperative action of cellobiohydrolases, endoglucanases, and beta-glucosidases (Lynd et al. 2002).

TASK 1: PREPARATION OF PRETREATED BIOMASS

Task Objective

The main objective of this task is to prepare pretreated samples (up to 100 kg) from the feedstock supplied by Weyerhaeuser using an innovative wet explosion pretreatment process to satisfy the specific needs of project partners.

Methodology

FS-01 and FS-03

Initially, the pretreatment parameters such as temperature, retention time, and oxygen loading were screened from a broad range of test conditions to identify best set of parameters for pretreatment of FS-01 and FS-03. The initial testing was performed using a 10 L batch pretreatment reactor equipped with a heating jacket. The best set of parameters was determined in terms of highest sugar release in enzymatic hydrolysis of the pretreated feedstock while the formation of degradation products was minimal. After the best pretreatment conditions have been identified, enzymatic hydrolysis was performed again using standard conditions, Temp=50 °C, pH=5.0, Enzyme loading CTec2=25 to 40mg EP (enzyme protein)/g cellulose + 10% HTec2 v/v. Finally the results were used to complete mass balances over the whole process for FS-01 and FS-03.

FS-10

A central composite design including three central points of the factors was used to design the experiments for identifying optimal pretreatment conditions of FS-10. A series of 17 runs with different operational conditions was suggested in order to generate regression reports of the factors and responses using response surface methodology. The wet explosion pretreatments of FS-10 were performed at temperatures ranged from 170-190 °C based on the preliminary experiments. Oxygen dosage used in this study varied in the range of 0.5-7.5% of DM (dry matter, w/w) with a retention time of 10-30 min. The array of pretreatment conditions were evaluated based on three factors, initial release of readily available sugars directly from pretreatment (+), enzymatic digestibility of the resulting cellulose (+), level of inhibitory compounds like HMF and Furfural, resulting from the pretreatment (-).

After the optimal pretreatment conditions have been identified, optimal pH for enzymatic hydrolysis of the pretreated FS-10 under optimized pretreatment conditions has been identified based on the total release of sugar as a direct result of enzymatic hydrolysis.

Finally the optimized conditions for the pretreatment of FS-10 and the optimal pH for enzymatic hydrolysis were used to conduct further study and determine the mass balance covering the whole process.

During this study, samples from all the different process steps have been coordinated and have been shipped to project partners as needed.

Results

During this study, the following biomass fractions were received from Weyerhaeuser: FS-01, FS-03, and FS-10. FS-10 was introduced at a later point and was not included in the initial screening. Due to challenges with the equipment on handling the comparatively large size feedstock, size reduction of the samples was necessary. Thus, all the biomass samples received (FS-01, FS-03, and FS-10) have been milled to a particle size of 1/16 inch (1.6 mm).

FS-01

Prior to any pretreatment work, the Douglas-fir (FS-01) was milled to 1/16” and the composition was determined as given in Table PPB-1.

Table PPB-1. Composition of Douglas-fir FS-01

Table 1. Douglas Fir (Raw composition)	
% Glucan	44.68
% Xylan	2.49
% Galactan	2
% Arabinan	2.09
% Mannan	11.82
% Lignin	29.58
% Extractives	5.03
% Acetate	2.34
% Ash	0.5
Sum	100.24

During preliminary work it was found that pretreatment of Douglas-fir is possible at a higher dry matter content than that of agricultural residues.

The initial pretreatment screening was done using a broad range of test conditions to identify best conditions for pretreatment of FS-01, see Table PPB-2.

Table PPB-2. Different pretreatment conditions and resulting sugar release

Pretreatment Conditions	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Galactose (g/L)	Arabinose (g/L)	Mannose (g/L)	HMF (g/L)	Furfural (g/L)	Acetate (g/L)
DF-PT-1 185C, 25m, 53 psi O2	1.16	9.55	7.27	5.7	2.18	13.97			
DF-PT-2 185C, 25m, 73 psi O2	1.2	11.53	8.23	6.56	2.52	16.44	2.798	0.522	5.238
DF-PT-3 175C, 25m, 53 psi O2	1.48	5.2	6.87	5.03	2.31	9.81			
DF-PT-4 175C, 25m, 73 psi O2	1.31	6.96	7.39	5.47	2.15	11.74			
DF-PT-5 195C, 25m, 53 psi O2	0.67	9.52	4.07	3.94	1.14	10.98			
DF-PT-6 195C, 25m, 73 psi O2	0.69	11.05	3.97	4.16	1.24	11.13			

From the initial sugar release data, pretreatment conditions were selected to perform further experiments in order to optimize enzyme loadings for enzymatic hydrolysis. The selected pretreatment conditions were 185 °C, 25 min, 73 psi, (7.50 % of DM oxygen)

To select the optimal loadings of enzymes and ratio of two commercial enzymes (CTec2 and HTec2) for enzymatic hydrolysis, the following loadings of enzyme mixtures have been evaluated which the other conditions for enzymatic hydrolysis remained constant (25% DM, 50°C, pH=5, and hydrolysis time=90h).

Parameters to be optimized were selected at:

- Enzyme loading CTec2 (20-60) mg EP/g cellulose.
- Enzyme loading HTec2 (10-50)% of CTec2 v/v

The results from this initial study can be found in Table PPB-3 and Figure PPB-2.

Table PPB-3. Summary of sugar release after enzymatic hydrolysis of pretreated FS-01

Table 3. Sugars (g/L)	G+C	Xylose	Galactose	Arabinose	Mannose
C (20mg/g glucan) + H (2mg/g glucan)	70.1	7.7	5.9	2.1	16.9
Cellulase only (20mg/g glucan)	68.8	8.5	7.0	2.0	19.4
Hemicellulase only (2mg/g glucan)	23.4	8.7	6.1	2.2	17.7
C (60mg/g glucan) + H (6mg/g glucan)	121.6	7.6	5.3	1.9	17.6
Cellulase only (60mg/g glucan)	83.8	6.1	2.8	0.7	12.2
Hemicellulase only (6mg/g glucan)	36.1	10.4	8.4	3.7	21.3
C (20mg/g glucan) + H (10mg/g glucan)	82.4	8.0	6.2	1.7	18.2
Cellulase only (20mg/g glucan)	68.8	8.5	7.0	2.0	19.4
Hemicellulase only (10mg/g glucan)	45.5	9.3	7.7	3.4	19.5
C (60mg/g glucan) + H (30mg/g glucan)	142.8	7.1	5.6	1.5	17.5
Cellulase only (60mg/g glucan)	83.8	6.1	2.8	0.7	12.2
Hemicellulase only (30mg/g glucan)	64.7	8.4	5.9	2.8	17.3
C (20mg/g glucan) + H (6mg/g glucan)	79.5	8.1	5.9	1.9	17.9
Cellulase only (20mg/g glucan)	68.8	8.5	7.0	2.0	19.4
Hemicellulase only (6mg/g glucan)	37.8	10.0	8.2	3.5	21.0
C (60mg/g glucan) + H (18mg/g glucan)	134.1	7.7	5.9	1.7	18.3
Cellulase only (60mg/g glucan)	83.8	6.1	2.8	0.7	12.2
Hemicellulase only (18mg/g glucan)	53.3	9.3	8.5	2.6	19.6
C (40mg/g glucan) + H (4mg/g glucan)	99.8	7.7	5.7	1.8	18.1
Cellulase only (40mg/g glucan)	95.7	8.1	5.7	1.7	18.6
Hemicellulase only (4mg/g glucan)	30.9	8.5	5.7	2.6	16.8
C (40mg/g glucan) + H (20mg/g glucan)	106.3	7.5	4.8	1.8	16.4
Cellulase only (40mg/g glucan)	95.7	8.1	5.7	1.7	18.6
Hemicellulase only (20mg/g glucan)	61.4	9.3	7.8	3.4	19.5
C (40mg/g glucan) + H (12mg/g glucan)	103.3	7.9	6.9	1.5	17.3
Cellulase only (40mg/g glucan)	95.7	8.1	5.7	1.7	18.6
Hemicellulase only (12mg/g glucan)	48.1	8.8	6.3	2.1	18.5

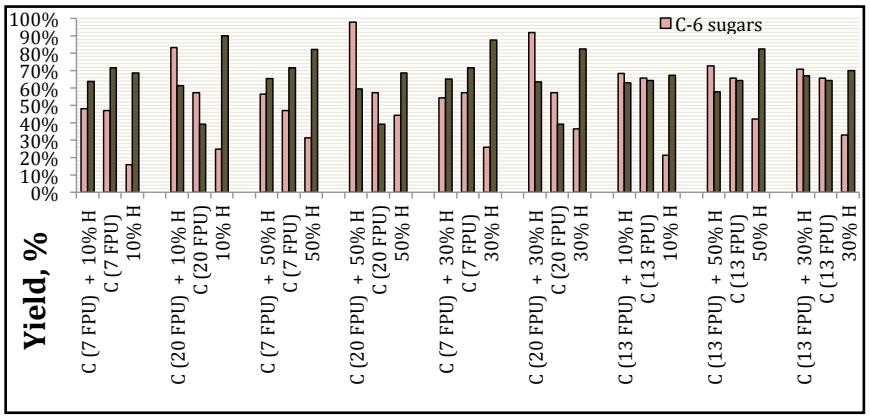

Figure PPB-2. Calculated yield obtained from optimization of enzymatic hydrolysis of FS-01 (Enzyme Loading, C=Cellulase, H=Hemicellulase).

From this optimization study the following conditions were selected for enzymatic hydrolysis, used for further studies and sample preparation using FS-01.

Selected conditions for enzymatic hydrolysis:

- Enzymatic hydrolysis conditions: 25% DM, Temp=50°C, pH=5, and time=90h.
- Enzyme loading: CTec2 45 mg EP/g cellulose + 25% v/v HTec2.

After the optimization of pretreatment conditions and of enzyme loadings for enzymatic hydrolysis, the mass balance for the process flow using FS-01 was completed, see Figure PPB-3. It is shown using optimal pretreatment conditions and an enzyme loading of 40 mg EP/g cellulose of CTec2 + 10%HTec2 of CTec2 volume.

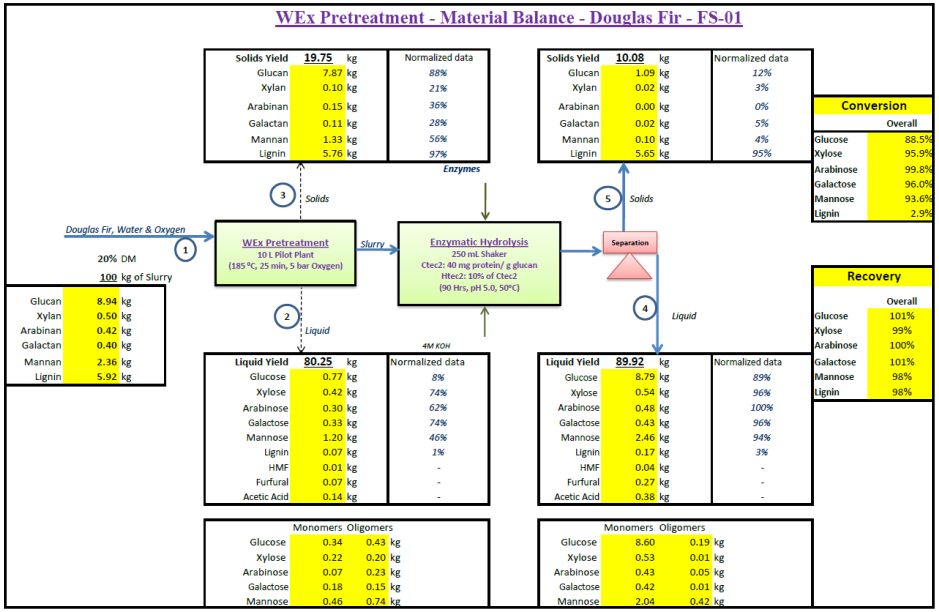

Figure PPB-3. Mass balance of FS-01 sample under optimized process conditions.

FS-03

Overall, FS-03 was found to be more challenging to perform both the size reduction and the pretreatment compare to FS-01 as well as enzymatic hydrolysis. This could be due to the nature and source of the feedstock as the structural integrity of different portion (root, bark, stem etc.) of Douglas-fir may vary significantly. Further, the source of biomass and the pre-existing conditions before harvesting of the feedstock can be different than that of FS-01.

The optimal pretreatment conditions found for FS-01 was used to subject FS-03 to the same process flow as performed on FS-01, this was done to enable a direct comparison of the resulting mass balances and evaluate if the physical differences observed would translate into differences in yields. As shown in Figure PPB-4, the conversion yields from FS-03 are significantly lower than the conversion yields found for FS-01 under the same process conditions.

The nature of the challenges presented by FS-03 pretreatment, compared to FS-01, was to some extent linked to differences in performance in the small 10L pretreatment system including mixing and ability to "flash" through the flash outlet of the smaller pretreatment system. Flashing the pretreated biomass is an important step towards high cellulose digestibility as the steam release within the biomass contributes in opening the lignocellulosic biomass matrix.

The pretreatment work performed in the smaller 10 L pretreatment system cannot be used to evaluate the overall pretreatment performance on FS-01 and FS-03 as the pretreatment system was not build to handle woody materials. To better evaluate the pretreatment performance on FS-03, more tests should have been performed in a larger pretreatment system as it was done for FS-10.

Due to limitations in FS-03 availability and the overall shift to FS-10 within the NARA project, no further optimization work was performed using FS-03.

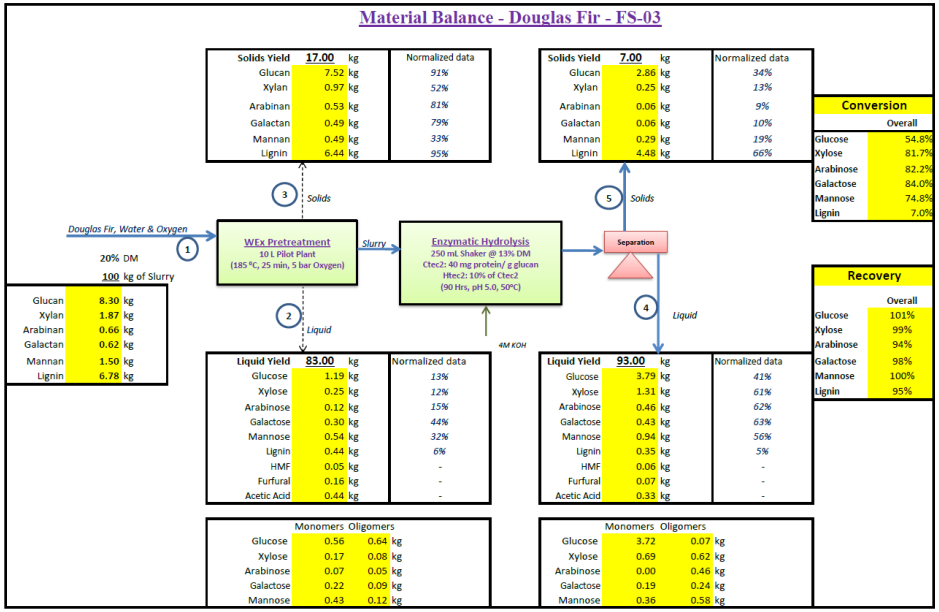

Figure PPB-4. Mass balance – FS-03 – not optimized

FS-10

The optimization of pretreatment parameters for the processing of FS-10 using wet explosion pretreatment involved extensive studies in order to increase the sugar yields from this biomass. This stepwise optimization includes sugar yield optimiza-

tion right after the pretreatment by screening the pretreatment conditions as well as yield optimization in enzymatic hydrolysis; this also contributes to the refining of the lignin by minimizing the cellulose content in the residual fiber.

The pretreatment optimization of FS-10 was based on an experimental design of 17 pretreatment conditions performed in pilot-scale. The process parameters such as temperature (T, 170-190 °C), oxygen dosage ([O_2], 0.5-7.5% of dry matter), and residence time (t, 10-30 min) were treated as factors to design the experiments. To assess process variability, three runs (8, 9, and 10) was used as a central point of process parameters i.e. T = 180 °C, [O_2] = 4% of DM, and t = 20 min as shown in Table PPB-4.

After receiving FS-10, the biomass was subjected to composition analysis, results shown in table PPB-5.

Table PPB-4. 17 test conditions screening for optimal pretreatment conditions for FS-10

Run	Time	Temp C	O2% of DM
1	10	170	0.5
2	10	170	7.5
3	20	170	4
4	30	170	0.5
5	30	170	7.5
6	10	180	4
7	20	180	0.5
8	20	180	4
9	20	180	4
10	20	180	4
11	20	180	7.5
12	30	180	4
13	10	190	0.5
14	10	190	7.5
15	20	190	4
16	30	190	0.5
17	30	190	7.5

Table PPB-5. Composition of FS-10 DM

Components	g/100g
Glucan	30.3%
Xylan	5.4%
Galactan	2.2%
Arabinan	1.4%
Mannan	8.8%
Acetyl	2.2%
Total lignin	46.22%
Ash	0.7%
Other*	2.8%

FS-10 was pretreated using a 100L pretreatment system and was initially subjected to a pretreatment condition screening consisting of 17 different test conditions as suggested by an experimental design using central composite design methodology, the test conditions are shown in Table PPB-6.

The samples from the 17 test conditions were then subjected to enzymatic hydrolysis at standard conditions for evaluation of cellulose digestibility. The results from the enzymatic hydrolysis were evaluated statistically and optimized towards four different scenarios, 1: Maximum total sugar, 2: Maximum total sugar with minimum inhibitory compounds, 3: Maximum glucose (cellulose digestibility), and 4: Maximum glucose (cellulose digestibility), with minimum inhibitory compounds, see Table PPB-6.

Table PPB-6. Results from pretreatment optimization of FS-10.

FS-10 Pretreatment optimization at 30%DM. Enzymatic hydrolysis performed using 40mg EP/g cellulose CTec + 10% HTec v/v in 100 ml @ 10% DM in 250 ml flasks (shake incubator)	
Maximum total sugar	23.1min 177.5C 7.5%O_2 (26.6 std1.7 g/l)
Maximum total sugar, Minimum HMF+Furfural	23.9min 170.5C 7.5%O_2 (25.8 std1.9 g/l)
Maximum glucose	30.0min 189.5C 7.5%O_2 (10.4 std0.8 g/l)
Maximum glucose, Minimum HMF+Furfural	26.5min 170.4C 7.5%O_2 (9.4 std0.8 g/l)

As can be seen in Table PPB-6, the results show that when high digestibility of the cellulose is desired, more severe pretreatment conditions are needed. This will result in the loss of hemicellulose sugars released at lower severity, mainly from hydrothermal hydrolysis of the hetero-xylan and later decomposition of the released sugar monomers.

The overall challenge for the pretreatment team is to maximize sugar yields from the biomass, from the observed trend. This is difficult to achieve in a single step pretreatment as the conditions needed for maximum yield of the hetero-xylan and the conditions needed for maximum cellulose digestibility can be significantly different and contradictive. Figure PPB-5 shows the resulting cellulose digestibility at 3 different oxygen loadings, (0.5, 4.0, and 7.5%).

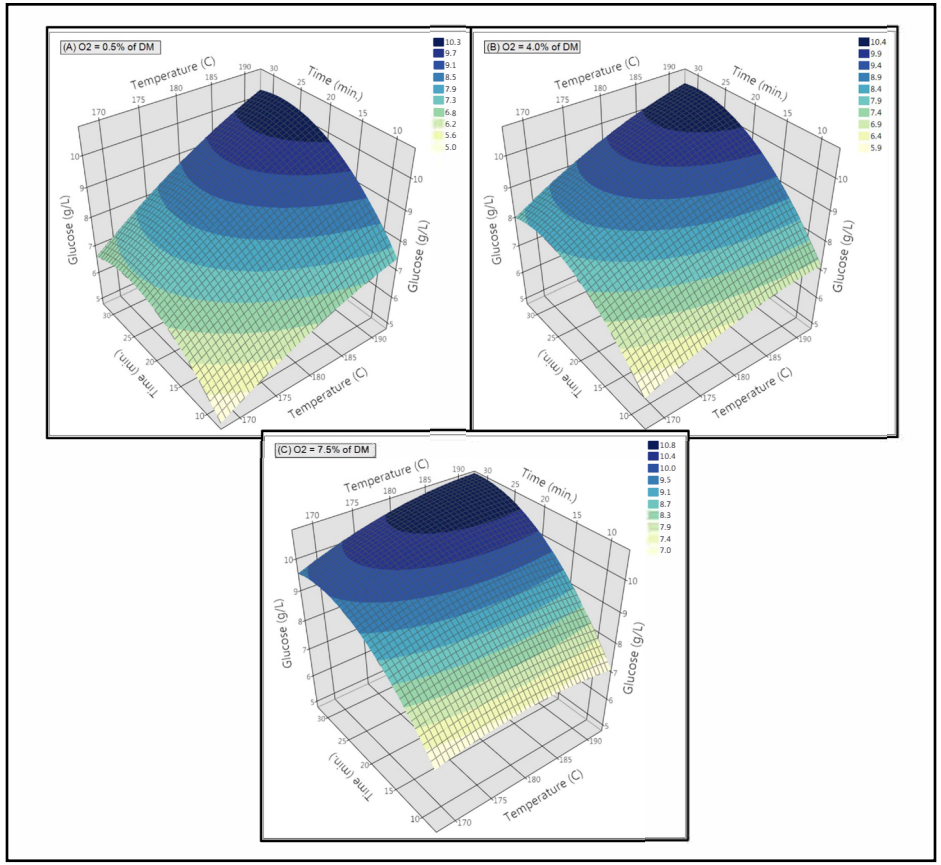

Figure PPB-5. Response surface models for glucose concentration after 72 hours of enzymatic hydrolysis (at pH 5.0, solids concentration of 10%, enzyme loadings of 40 mg EP/g glucan) of the pretreated samples at different oxygen loading (%, w/w); (A) Oxygen loading of 0.5% of DM, (B) Oxygen loading of 4.0% of DM, (C) Oxygen loading of 7.5% of DM.

The hetero-xylan content of FS-10 is significant (23.91% of DM). Therefore it was determined that refining the biomass at a comparatively lower severity and obtaining a residual fiber, rich in cellulose and lignin would be desirable, before a high severity pretreatment would be used to access the cellulose fibers, thus a two-step pretreatment process would be beneficial in order to achieve desirable yields in the overall process.

From analysis of the total sugar concentration in the liquid fraction as obtained after pressing the pretreated slurry, optimal conditions were identified in terms of maximum sugar release by pretreatment only, targeting the hetero-xylan sugars.

From the collected data a prediction profiler was built to assess the response of the parameters and the optimal conditions were found by maximizing the desirability of achieving maximum total sugar in the liquid. The prediction profiler also outputs a predicted sugar concentration at predicted optimal conditions. From this plot it is found that a high total sugar concentration of 69.9 ± 3.6 g total sugar/l is achievable using lower severity pretreatment conditions at 10 min, 182.6 °C, and 5.53% oxygen loading, at 30% DM, see Figure PPB-6.

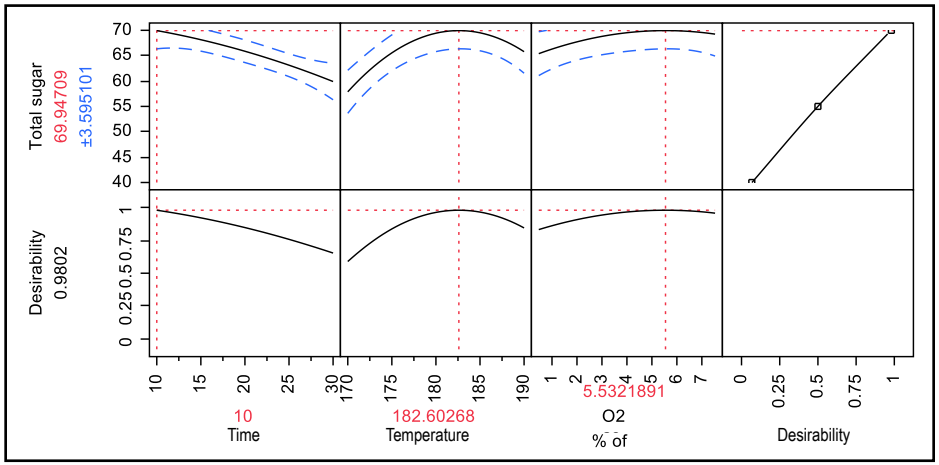

Figure PPB-6. Prediction profiler indicating the pretreatment conditions needed to archive maximum total sugar concentration in the raw pretreatment liquid.

It is important to note that this data was derived from the pretreatment performed at 30% DM and that pretreatment of FS-10 has been successfully performed at dry matter up to 40% (data not shown). Performing the pretreatment at higher DM will increase the total sugar concentration in the liquid significantly. This liquid fraction containing high sugar concentration can be obtained directly after pretreatment and without the use of any expensive enzymes! Conducting enzymatic hydrolysis at 40% dry matter will, however, can be unfavorable due to the known challenges and limitations of running the enzymatic hydrolysis at a high DM content. The enzymatic hydrolysis would preferably be carried out at or close to 20% DM, thus lowering the resulting total sugar concentration significantly. It is important to note that the comparison made here is merely based on the concentration of total soluble sugar. However, sugars obtained directly after pretreatment and without enzymes will have a far lower production cost than the ones produced after enzymatic hydrolysis.

This upfront sugar extraction combined with a simple back wash solution will not only increase the overall yield of fermentable sugars significantly, but also refine the cellulose/lignin complex, preparing the fiber for a more targeted high severity pretreatment, where maximizing the cellulose digestibility could be more focused.

For better understanding the factors effect on sugar extraction during pretreatment, three surface profiles was generated from the model to visualize the isolated effect of Time vs. Temp, Oxygen vs. Time, and Oxygen vs. Temp, (Figure PPB-7).

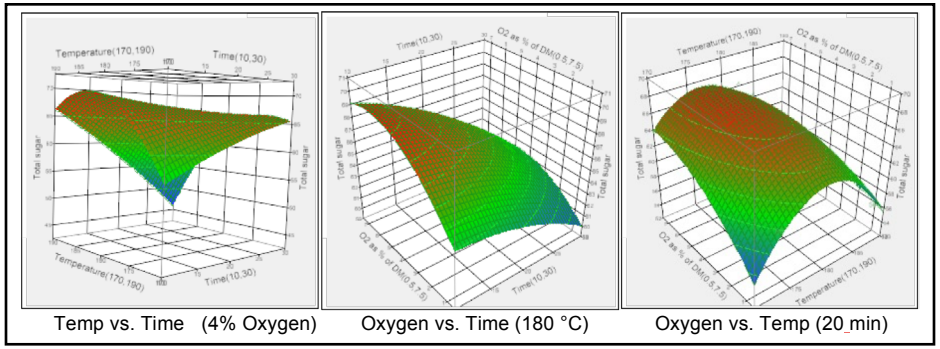

Figure PPB-7. Factors effect on FS-10 pretreatment for total sugar extraction in the liquid fraction of pre-treated slurry.

From Figure PPB-7

Time vs. Temp (4% Oxygen) – It can be seen that at low temperature, longer retention time is desirable and that longer retention time does not result in an increase of the total sugar concentration. Increasing the temperature at a low retention time will dramatically increase the total sugar concentration to a certain point, after which the total sugar concentration starts to drop. This drop is most likely the result of hydrothermal degradation of monomeric sugar released from the hetero xylan. This observation was also confirmed by an increase in concentration of both HMF and furfural. It is also observed that the plot does not have a top point and therefore the optimum for time and temperature cannot be identified in this plot, but the tendency shows that higher temperature and shorter retention time will increase the total sugar concentration in the press liquid.

Oxygen vs. Time (180 °C) – As displayed in Figure PPB-7 (Oxygen vs. Time), longer retention time results in lower total sugar concentration. Low oxygen dosage also results in low total sugar concentration. The trend suggests that the highest sugar concentration can be obtained by reducing the retention time and increasing the oxygen loading in the pretreatment.

Oxygen vs. Temp (20 min) – It can be observed that at low temperature, increasing oxygen loading will have a significant effect on increasing total sugar concentration. It can also be seen that at center point of the temperature (ca.180 °C), increasing oxygen loading will have a significant positive effect on total sugar concentration, the maximum total sugar concentration from this plot is found at the approximate conditions of 177 °C, 20 min (fixed), 6% Oxygen.

In order to maximize the sugar yield in enzymatic hydrolysis, the optimal pH was determined by testing enzymatic hydrolysis of the pretreated sample under different pH ranging from 4.75 to 6.50. This pH optimization for enzymatic hydrolysis is particularly important as the interactions between the different enzymes and pretreated biomass can be significantly different at different pH.

The pretreatment conditions used to perform the pH optimization was selected based on maximum cellulose digestibility (190 °C, 30 min, 7.5% Oxygen, see Table PPB-6) and was performed at standard conditions (50°C, 72 hours, and 40 mg EP/g cellulose CTec + 10% HTec v/v) 100 ml @ 10% DM in 250 ml flasks (shake incubator).

Effect of pH on hydrolysis using commercial enzymes was significant and pH 5.5 was found to be optimal which is higher than the pH 5 as normally used, (Figure PPB-8).

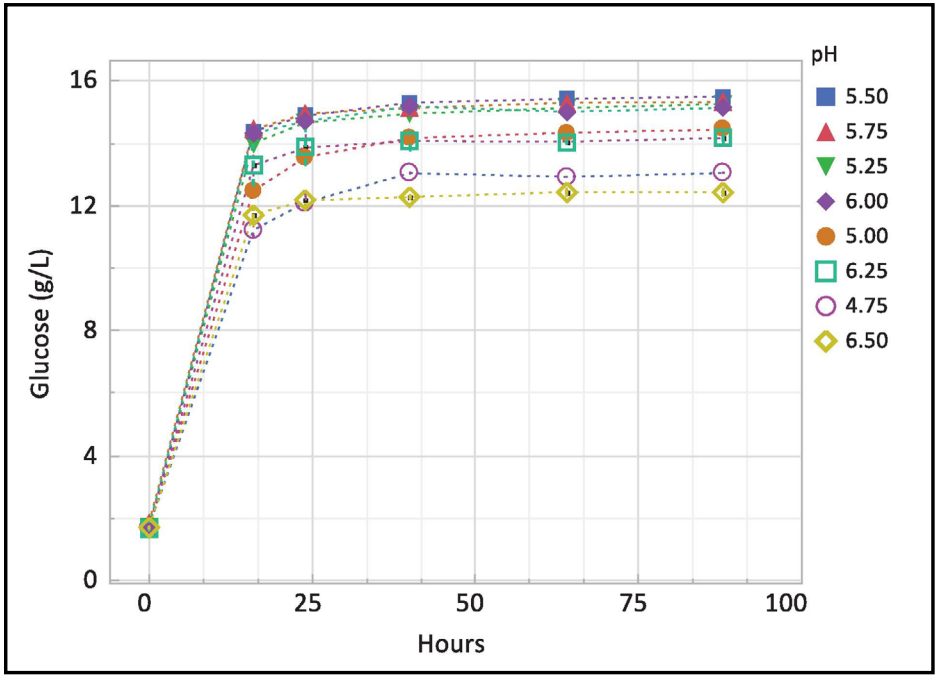

Figure PPB-8. Effects of pH on the hydrolysis of the pretreated sample under optimal pretreatment conditions for maximum cellulose digestibility (190 ºC, 7.5% O_2 loading, and 30 minutes residence time).

The optimal conditions for the pretreatment of FS-10 were selected based upon the maximum cellulosic sugar yield in the enzymatic hydrolysis (Biswas et al., 2015). To improve the hydrolysis of the pretreated sample under optimal conditions further, an enzymatic loading of 80 mg EP/g cellulose (of which 70% CTec2 and 30% HTec2) was used. The run under the pretreatment conditions of 190 °C, 30 min, at 7.5% O_2 of DM (run#17) was selected to be the optimal and to better understand the process using the pretreatment conditions an overall mass balance is determined as depicted in the Figure PPB-8. As shown, 63.3% of the glucan has been converted during the enzymatic hydrolysis and can be recovered as monomers with an overall recovery of 99.9%. The recalcitrance of Douglas-fir as softwood biomass for bio refineries has previously been reported. Dilute acid hydrolysis, for instance, converted less

than 50% of the cellulose (Geleynse et al., 2014) with hydrolysis yields of cellulose less than 50%. Especially the forest residuals like Douglas-fir (FS-10) as tested in this study showed a higher degree of recalcitrance during enzymatic hydrolysis.

Lignin deposition during hydrothermal pretreatment on cellulose surface potentially limits the enzymatic hydrolysis (Li et al., 2014), however this effect could possibly be reduced if the solid fraction is grinded after pretreatment, before hydrolysis. The higher lignin content in the solid fraction of WEx treated FS-10 sample could be due to the incomplete hydrolysis of the solids during the two steps acid hydrolysis performed during compositional analysis. Although the mass for glucan and lignin after enzymatic hydrolysis appeared to be satisfactory as the enzymatic hydrolysis was carried out in the whole slurry without any solid-liquid separation. The glucan and lignin recovered after enzymatic hydrolysis was 99.9% and 96.3%, respectively. Further, the overall recovery of xylan and mannan was 69.2% and 76.0%, respectively. Some degradation products such as furfural and HMF were initially formed from the degradation of xylose and mannose, as shown in the mass balance (Figure PPB-9). Although the mass of the degradation products found in this study was determined, the mass of furfural and HMF was not added to the xylose and mannose in the overall recovery calculation, as those degradation products could result from degradation of any C5 and C6 sugar. However, the furfural and HMF concentrations in the pretreatment hydrolysate were relatively low. Our study suggests that only 30.8% and 24.0% of the xylose and mannose, respectively, would be lost under these more severe pretreatment conditions. Previous studies carried out on FS-03 (a similar forest biomass residues) using the SPORL pretreatment (Leu et al., 2013), showed that 70% and 50% of xylose and mannose, respectively, were lost. The lack of mass balance closure for such pilot-scale studies is attributed to the degradation of sugars during this type of pretreatment.

We are currently studying the biomass lignin portion left behind our pretreatment process to understand its characteristics and potential for producing high-value products. Initial characterization studies have indicated that the biomass lignin does not undergo any major structural transformation, as is the case with traditional sulfuric acid or sulfite based pretreatments, thus making this lignin ideal for co-products research. Literature studies have indicated that the biomass lignin structure opens up during traditional dilute acid pretreatment processes and re-condenses and re-distributes itself after the pretreatment making it difficult to activate and convert into high value co-products. These studies have also indicated that the re-condensation and re-polymerization of the lignin compounds over the cell wall adversely affects the cellulose enzyme activity, thereby, affecting the overall process and yields. However, such problems were irrelevant with wet exploded biomass lignin as no such chemicals are used in this process. The characterization studies of lignin obtained after wet explosion pretreatment have also indicated that hydroxyphenyl- or H-form of lignin was converted to syringyl- or S-form of lignin after wet explosion pretreatment making the process more amenable for co-product production from the biomass lignin.

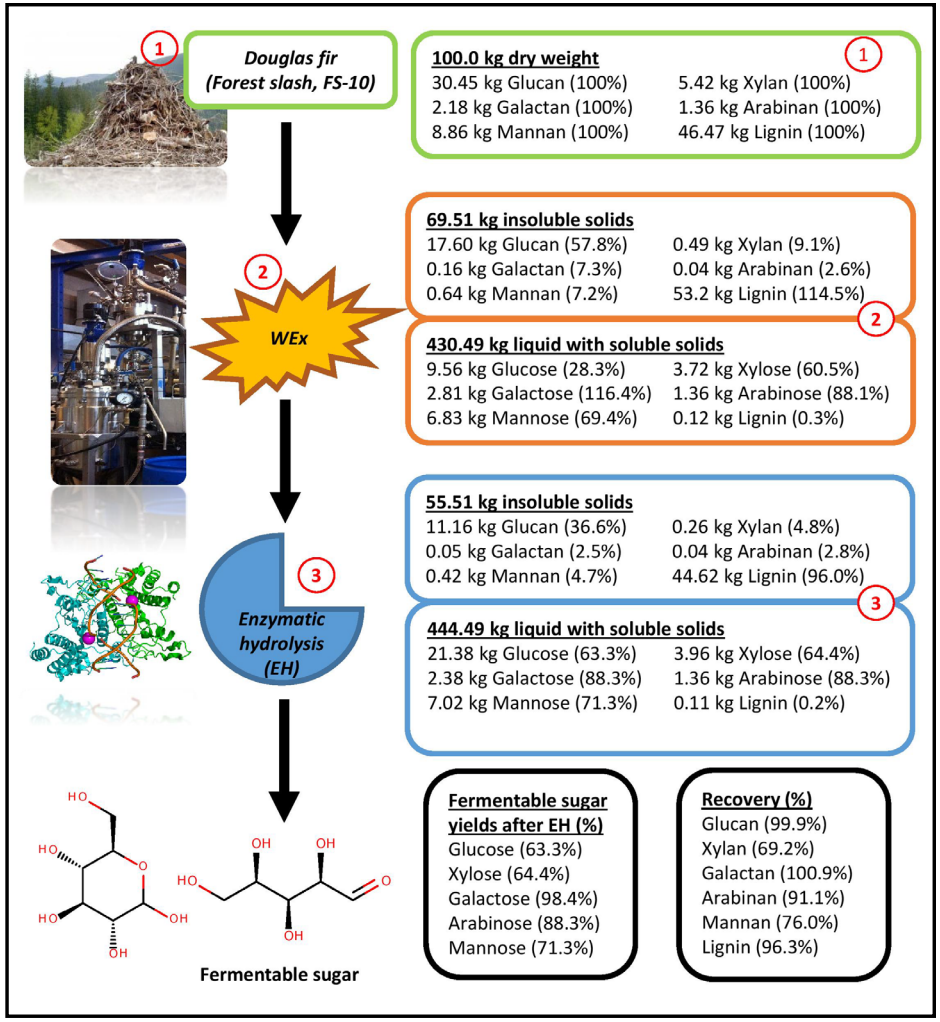

Figure PPB-9. Overall mass balance of the optimal run (190 ºC, 7.5% O_2 loading, and 30 minutes residence time.

From the above discussions, it is evident that wet explosion pretreatment at its optimized conditions can effectively breakdown lignocellulosic woody biomass (including forest slash) with effective C5 and C6 sugar conversions while providing an ideal and clean biomass lignin for valuable co-product production.

NARA OUTPUTS

Samples for partners

Table PPB-7. Samples for partners

Gevo	3/14/2012	FS-01-Hydrolysate
Gevo	3/14/2012	FS-01-PT sample
Gevo	12/26/2012	FS-03-Hydrolysate
Gevo	2/18/2013	FS-03-Hydrolysate
Weyerhaeuser	2/18/2013	FS-03 Lignin
Weyerhaeuser	4/16/2013	FS-03 Lignin
Gevo	11/18/2013	FS-10 hydrolysate
Weyerhaeuser	11/25/2013	FS-10 Lignin fiber
Gevo	01/25/2014	FS-10 Pretreated slurry
Gevo	01/29/2014	FS-10 Pretreated slurry

Research Presentations

1. Srinivas, K., Oliveira, F., Teller, P., Helms, G. and B. Ahring. 2015. Characterization and optimization of alkaline wet oxidation of biorefinery lignin obtained from pretreated forest slash, Pacifichem, Honolulu, Hawaii, December 15-20.

2. Presentation at the 35 SBFC, Portland, Oregon. April 29 – May 2, 2013. Pretreatment of Forest Slash using Wet Oxidation.

3. Ahring, B., D. Rana, V. Rana K. Srinivas and P. Teller. 2012. Breaking the barriers of Douglas fir softwood to biofuels using wet explosion pretreatment. Poster presentation at NARA 2012 Annual Meeting, Missoula, MT, Sept 13-14, 2012.

Refereed Publications

1. Rajib Biswas, Philip J. Teller, Birgitte K. Ahring. 2017. Optimization of sugar production from hybrid poplar sawdust using wet explosion pretreatment. *Biomass Bioenergy*, submitted manuscript.

2. Srinivas, K., Oliveira, F., Teller, P., Helms, G. and B. Ahring. 2016. Oxidative degradation of biorefinery lignin obtained after pretreatment of forest residues of Dogulas fir. *Bioresour. Technol.*, 221, pp. 394-404.

3. Biswas, R., Teller, P. and B. Ahring. 2015. Pretreatment of forest residues of Douglas fir by wet explosion for enhanced enzymatic saccharification. *Bioresour. Technol.*, 192, pp. 46-53

NARA OUTCOMES

The outcomes from the study using wet explosion pretreatment to deconstruct biomass towards fuel and chemicals production had significant intellectual merit. Traditional pretreatment techniques use harmful chemicals such as (but not limited to) sulfuric acid, which results in production of modified biorefinery lignin streams that directly have limited applications and require further purification and modification for production of high-value products. Such processes used in fuel production also result in "dirty" mixtures that are hard to ferment and can be generally inhibitory to several microorganisms resulting in either limited optimized applications or increase in process steps to further clean these streams resulting in higher costs and lower efficiencies. Unlike such processes, wet explosion pretreatment uses no harmful chemicals during biomass pretreatment other than oxygen and has been sufficiently optimized towards effective biomass depolymerization for use in fuel production. The biorefinery lignin stream is also unmodified and in its "native" form after pretreatment and can be used to produce chemicals (see Appendix I in this report for more information). As part of the process, Weyerhauser did elemental analysis on the biorefinery lignin obtained from different biomass pretreatment processes tested as part of the NARA project and indicated wet exploded lignin to have significant impact for co-products (Table PPB-8). The data from this work was also used in application of several research proposals submitted to NSF, DOE, USDA and local agencies such as WA-JCATI.

Table PPB-8. Elemental composition of lignin from different biomass pretreatment

Sample	% C	% H	% N	% O	%S
Forest Residual Lignin	61	6.0	0.07	32.9	0.032
Wet Exploded Lignin	64.9	5.3	0.96	28.7	0.096
Mild Bisulfite Lignin	63.3	5.7	1.01	28.9	1.13
Mild Bislufite Lignosulfonic acid	52.1	4.8	0.36	34.7	8.10

Apart from the better understanding of wet explosion pretreatment in effective biomass depolymerization, efforts were also taken to discuss and educate local community in the different processes that form part of "biorefinery applications". As a part of this effort, Dr. Ahring (PI) participated in a summer program that allowed students from Walla Walla community college to visit the laboratory and pilot plant operations at Washington State University, Tri-Cities to practically study the various biorefinery processes towards fuel and chemicals production. Further details about the program can be found at https://news.wsu.edu/2015/08/03/new-course-offers-hands-on-training-in-bioproducts/.

FUTURE DEVELOPMENT

Upfront biomass downsizing

Due to challenges in handing the biomass using the available process equipment, it was necessary to reduce the size of the biomass particle to 1/16 inch (1.6 mm). It is possible that the upfront downsizing could have had a negative effect on the overall process performance, especially the enzymatic hydrolysis, due to the possible post pretreatment effect such as lignin re-condensing on the accessible cellulose surface, thus making it inaccessible for the enzymes.

Post pretreatment size reduction

It is evident that biomass particle size will have a great impact on overall process performance, one of the challenges is that the biomass lignin will redistribute after pretreatment and can therefore interfere with the overall process performance, especially during enzymatic hydrolysis. Future development should therefore be directed towards post pretreatment process steps like disk refining, which will dramatically reduce the average biomass particle size as well as re-condensation of the lignin, thus enzymatic hydrolysis can be improved without adding unnecessary process cost.

By-products

Refined lignin resulting from the WEx pretreatment should be further investigated for potential bio-products, the WEx derived lignin is pure sulfur free lignin and could potentially be used for a broader array of by-products. Unlike most comparable pretreatment methods where sulfuric acid or sulfite is added, the WEx pretreatment only uses oxygen. The lignin product is therefore sulfur free and very different from the lignin fiber deriving from the sulfur containing pretreatments. Therefore the lignin from the WEx pretreatment should be considered as a valuable byproduct vs. a toxic waste stream. (Refer to Section I or Appendix for further information)

Maximizing yields

Fermentation of the press liquid from the first step of pretreatment should be further investigated as a raw material for fuel production. The sugar content of the upfront press liquid has shown to reach up to 70 g/l from pretreatment at 30% DM, and this could potentially be increased up to 100 g/l using higher biomass concentrations during pretreatment. The stream will have low inhibitory levels and this sugar stream could be generated without the use of any enzymes, offering a low cost sugar stream.

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APPENDIX

As indicated in section "Future Development" in this report, the biorefinery lignin stream obtained from wet explosion pretreatment of harvested forest residues such as FS-10 can be considered as a "valuable" feedstock for production of several chemicals that can either be used in further catalytically upgraded to fuels or sold as-is (after sufficient purification, of course) to supplement for the cost of fuel production from lignocellulosic biomass. However, such a process requires complete information about the biorefinery lignin stream obtained after wet explosion pretreatment. As part of the study done in NARA, our team investigated the biorefinery lignin stream obtained after wet explosion pretreatment under optimized pretreatment conditions for further wet oxidation to produce high-value chemicals such as acetic acid, formic acid, lactic acid, vanillin, syringaldehyde and 4-hydroxybenzaldehyde. Initially, compositional analysis was done on the FS-10 lignin stream and compared with original FS-10 before pretreatment (as shown in Table PPB-app.1). It can be seen from Table PPB-app.1.1 that the biorefinery lignin stream still contained about 23wt% cellulose while most of the hemicellulose was removed during wet explosion pretreatment. While most research groups focus on further purifying the lignin stream resulting in increased process costs, the goal of our study was identify potential for this biorefinery lignin stream to produce high-value chemicals.

Table PPB-app.1. Compositional analysis of biorefinery (FS-10) lignin

Component	Composition of FS-10(%)	Composition of Wet-Exploded FS-10 (%)
Cellulose	30.3	23.1
Xylan	5.40	1.84
Galactan	2.20	0.19
Arabinan	1.40	0.00
Mannan	8.80	2.21
Ash	0.70	0.30
Lignin	46.2	56.8
Extractives	2.80	11.8

Solid-state NMR (Figure PPB-app.1) was done on the biorefinery lignin to qualitatively predict the composition of both sugars and lignin components. The solid-state NMR for the untreated FS-10 had to be done at a lower frequency to obtain comparable spectra with the biorefinery lignin. For the convenience of the readers, the resonance band assignments were presented in Figure PPB-app.1. It was seen from the solid-state NMR spectra that the acetate methyl group peaks usually occurring around 173 ppm was significantly reduced in the biorefinery lignin when compared to original FS-10. However, an increase in the methoxy groups in G & S units (as can be seen in Figure PPB-app.1) supported previous results indicating that wet explosion pretreatment leads to an in-situ methoxylation of the hydroxyphenyl- (H-) and guaiacyl- (G-) components present in lignin resulting in generation of syringyl- (S-) like structures (Rana et al., 2015). The solid-state NMR spectra did, however, not provide more qualitative information on the lignin components present in the biorefinery lignin, mainly since they were considerably lower when compared to the sugar-based peaks and hence, FTIR analysis was done on the biorefinery lignin for further understanding (Figure PPB-app.2).

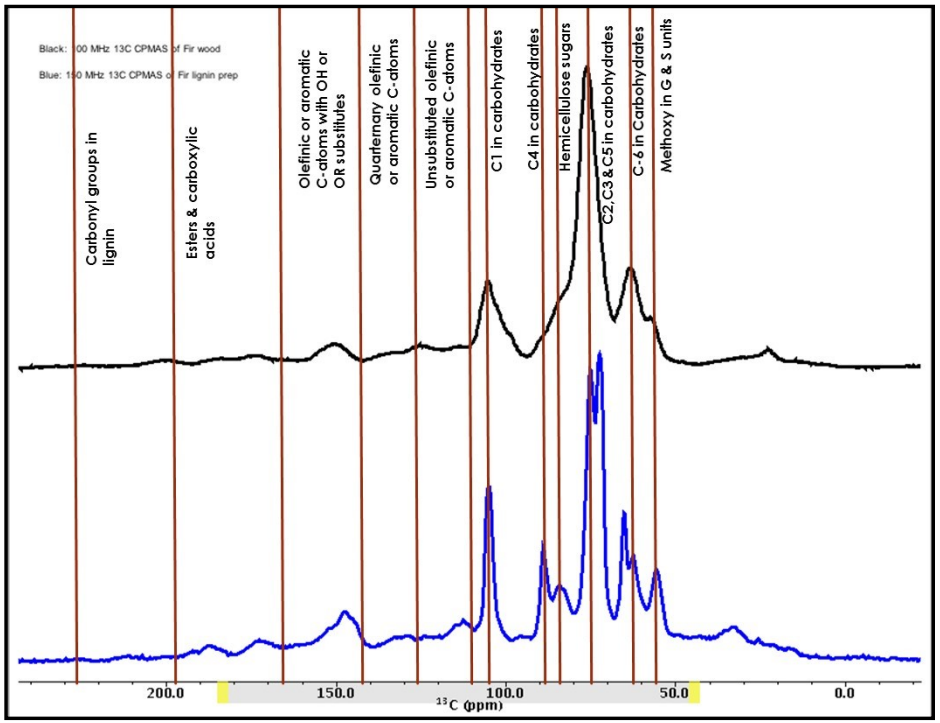

Figure PPB-app.1. Solid-state NMR spectra of untreated FS-10 and biorefinery lignin stream; black line showing ^{13}C CPMAS spectra at 1MHz for FS-10 and blue line showing ^{13}C CPMAS spectra at 1MHz for biorefinery lignin.

In FTIR spectra shown in Figure PPB-app.2., it was found that the intensity at 1467, 1318 and 1220 cm^{-1} bands non-negligible which are commensurate with prominent bands in woody biomass (Pandey, 1999). However, normalized intensity at 1270 cm^{-1} and 1510 cm^{-1} (1.07 and 1.00 respectively) was used to indicate that the biore-

finery lignin was predominantly softwood lignin in nature (Pandey, 1999). Unlike FTIR spectra of pure softwoods, the biorefinery lignin also showed significant peak intensity at 1318 cm^{-1} ,which is related to the C-O stretching of the syringyl ring and is common only among hardwoods (Lin and Dence, 1992). Previous studies have indicated that the G-type lignin absorbs in both 1270 and 1230 cm^{-1} bands, S-type lignin components absorbed mostly in the 1230 cm^{-1} bands (Pandey, 1999). The S/G ratio of the biorefinery lignin was found to be 0.88 by comparing the FTIR absorbance at 1270 cm^{-1} and 1225 cm^{-1} respectively. The small peak shift for the syringyl units was caused due to the mixed nature of biomass. Apart from the FTIR spectra for the lignin-based components, there were significant peaks for the sugar components present in the biorefinery lignin primarily at 1030 cm^{-1} (related to primary and secondary alcohols from cellulosic units) and above 3000 cm^{-1} (related to OH- bonds in cellulosic units). Peak intensities were also observed for C-H stretching of methyl and methylene groups associated with cellulosic units at 2905-2933 cm^{-1}, which can be attributed to methoxylation of sugars during wet oxidation (Guay et al., 2001). Further details on the study can be found at Srinivas et al. (2016).

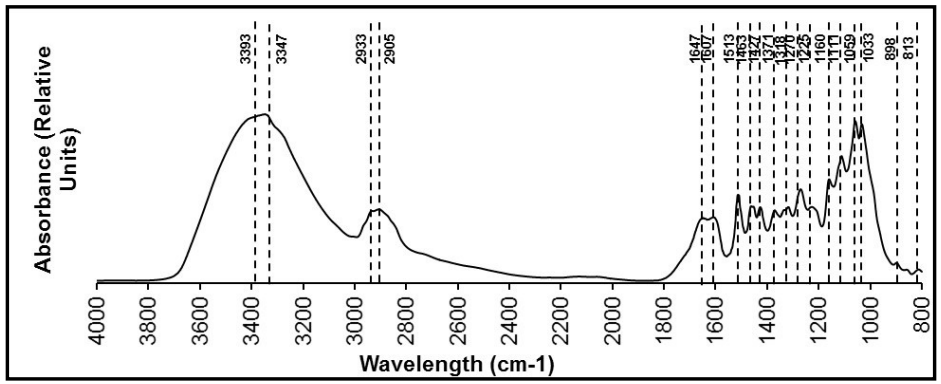

Figure PPB.app.2. FTIR spectra of biorefinery lignin stream showing spectral bands pertaining to component peaks present in biomass.

In the next part of this study, the biorefinery lignin (dried and milled) was mixed with a hydrogen peroxide solution in stainless steel tubes and placed in a preheated fluidized sand bath. Initial experiments were performed without addition of alkali and process conditions tested include 10% solids loading, temperatures from 180 to 300°C and 5 and 15 min residence times. Second set of experiments was performed through addition of alkali at 1 g/g dry biorefinery lignin (11.7 wt% alkali) and 1.5 g/g dry biorefinery lignin (17.4 wt% alkali) at the similar experimental (temperature and residence time) conditions as specified previously. After wet oxidation, the stainless steel tubes were placed in an ice-water bath until the tubes were cooled to below room temperature. The wet oxidized samples from the reaction tubes were then transferred to centrifuge tubes and the reaction tubes were washed with known volume of water. The wash water was added to the centrifuge tubes and mixed before centrifuging at 10,000 rpm for 5 min to separate out the solids from the liquid. In case of alkaline wet oxidation, the samples were neutralized with hydrochloric acid (1N) before centrifugation. The liquid samples were appropriately diluted and analyzed using HPLC while the solids were analyzed using FTIR. The results obtained from the study was separated into three main parts (as summarized from Srinivas et al., 2016):

1. **Effect of alkaline wet oxidation on sugar oxidation products such as glucose, lactic acid and formic acid:**
   While glucose and formic acid can be produced as a result of thermal degradation of cellulose, the primary oxidation product from cellulosic component in the biorefinery lignin would be lactic acid. From the study, wet oxidation under alkaline conditions resulted in greater yields for glucose, lactic acid and formic acid with maximum yields of 129±6.49 mg glucose/g dry biorefinery lignin at 230°C; 116±1.71 mg formic acid/g dry biorefinery lignin at 250°C; and 124±0.01 mg lactic acid/g dry biorefinery lignin at 300°C with an alkali loading of 1 g/g biomass (11.7 wt% alkali) and residence time of 15 min. Compared to the literature that uses higher oxygen loading than 7.5% used in our experiments, the results showed slightly higher lactic acid yields at similar temperature but higher residence times. However, No significant difference in the yields of glucose, formic acid and lactic acid could be observed as a function of alkali loading.

2. **Effect of alkaline wet oxidation on lignin oxidation products such as vanillin, syringaldehyde and hydroxybenzaldehyde:**
   As can be seen from Figure PPB-app.3 (a) (i) & (ii), there was no significant effect of residence time and alkali loading on the vanillin yield from the alkaline wet oxidation of biorefinery lignin. There was, however, a significant effect of temperature with maximum yield of 3.85±0.24 mg vanillin/g dry biorefinery lignin at 230°C, 11.7 wt% alkali loading and 15 min residence time. It was also seen that there was no significant difference between vanillin yields at 230 and 250°C and different residence times when alkali loading was 11.7 wt%. However, there was a significant effect of residence time and temperature on the hydroxy benzaldehyde and syringaldehyde yields with maximum values of 2.01±0.02 mg/g dry biorefinery lignin and 1.32±0.02 mg/g dry biorefinery lignin respectively at 280°C, 11.7 wt% alkali loading and 15 min residence time. Since there is only around 56.8 wt% of lignin in the biorefinery lignin feed, the theoretical maximum yields of vanillin, syringaldehyde and hydroxybenzaldehyde was calculated as 6.79, 2.32 and 3.54 mg/g dry pure lignin respectively. While vanillin yields as high as 12 wt% has been shown from soft wood lignin (Fache et al., 2016), the recalcitrance of the biorefinery lignin from mixed forestry residues should be taken into consideration along with the high temperatures tested in the study, which could lead to a decrease in expected vanillin yields. Another reason for lower yields of lignin oxidation products, when compared to the afore-mentioned studies, is that they has used a lower solids loading (≤ 6 wt%) when compared to the current study.

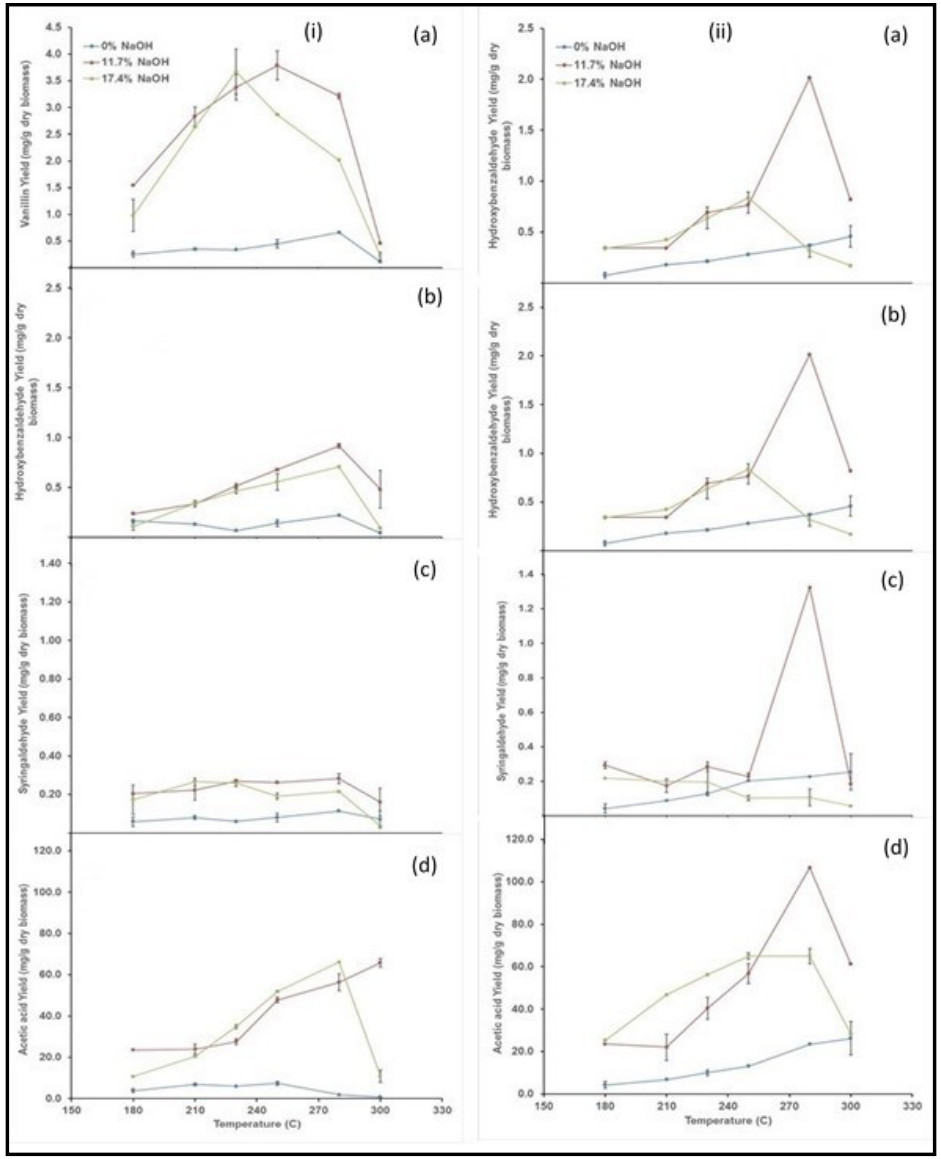

Figure PPB-app.3. Variation in concentration (mg/g dry biomass) of lignin oxidation products (a) vanillin, (b) hydroxybenzaldehyde, (c) syringaldehyde, and (d) acetic acid as a function of temperature at different NaOH loadings and residence time of (i) 5 mins and (ii) 15 mins. (● – 0wt% NaOH; ▲ – 11.7wt% NaOH; ■ – 17.4wt% NaOH)

As can be seen from Figures PPB-app.3.(b) (ii) and (c) (ii), the hydroxybenzaldehyde and syringaldehyde yields increased significantly above 250°C followed by a decrease at 300°C probably due to thermal degradation. This sudden increase in yields above 250°C was also accompanied by a significant decrease in the vanillin yields (shown in Figure PPB-app.3. (a) (ii)). The thermal degradation of vanillin above 250°C can be attributed to the increased severity of alkaline wet oxidation conditions. An increase in the alkali loading to 17.4 wt% showed a significant decrease in vanillin yields as a function of temperature above 210°C and residence time of 15 mins, which can again be attributed to the increased severity of the alkaline wet oxidation. Hence, reduced temperatures and higher residence times (above 15 min) would have sufficiently favored lignin conversion to vanillin in the current study. However, in absence of alkaline conditions, vanillin yields from wet oxidation of biorefin ery lignin increased with an increase in temperature until 280°C but the yields, as expected, were considerably lower than that obtained under alkaline conditions. The selectivity toward oxidation of biorefinery lignin to vanillin can be considerably improved through the optimizing temperature, residence time and the use of a catalyst. Since hardwood content in the forestry residue was lower, significant syringaldehyde yields were not expected using the current biorefinery lignin. Apart from vanillin, syringaldehyde and hydroxybenzaldehyde, other G-, S- and H-type lignin oxidation products such as catechol (H), 4-ethyl guaiacol (G), 4-propyl guaiacol (G), homovanillyl alcohol (G), homovanillic acid (G), syringol (S) and propylveratrole (S) was also found to be produced and was characterized through GC-MS analysis.

3. **Effect of alkaline wet oxidation on acetic acid:**

While there have been studies that have discussed the production of acetic acid primarily from the oxidation of cellulosic components (Jin et al., 2005), there has also been other studies that show the wet oxidation of lignin compounds resulting in acetic acid. Theoretical calculations indicated that, at optimized conditions, only around 7.85 mg of acetic acid/g dry biorefinery lignin was produced from cellulosic substrates while liquid phase analysis after alkaline wet oxidation indicated that almost 107 mg of acetic acid was produced per g dry biorefinery lignin. The excess acetic acid would have to be produced from the wet oxidation of the lignin components since all the water-soluble extractives present in the biorefinery lignin was sufficiently removed during water washing before wet oxidation and the concentration of hemicellulosic components was found to be minimal in the biorefinery lignin. Incorporating the small amount of hemicellulosic components such as xylose in the calculations did not significantly alter the amount of acetic acid produced from the lignin components. Due to the significant differences in the chemical reactivity of the components present in biorefinery lignin, the theoretical calculations are only an approximate qualification to indicate that under the optimal conditions in this study, significant amounts of lignin oxidation products produced acetic acid at higher temperatures (>250°C). However, these theoretical calculations also showed that the cellulosic components present in the biorefinery lignin were the primary sources for acetic acid production

when compared to lignin components. Since acetic acid has significant applications in chemical and fuel sectors, an increased acetic acid yield from the biorefinery lignin (from both sugar and lignin components) is more desirable when compared to a pure lignin stream.

Based on the study, the overall mass balance for conversion of biorefinery lignin stream after wet explosion pretreatment using alkaline wet oxidation is shown in Figure PPB-app.4.

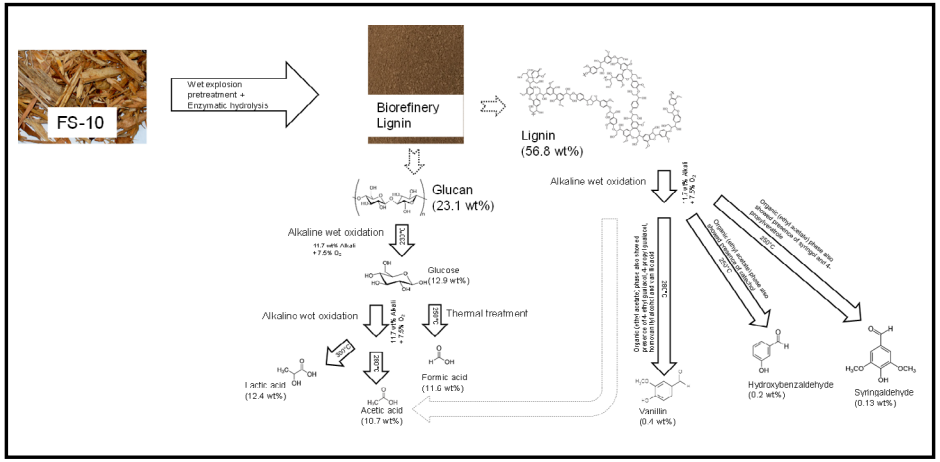

Figure PPB-app.4. Overall process reaction scheme showing the alkaline wet oxidation of biorefinery lignin to bioproducts.

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