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Evaluation of Mountain Beetle-Infested Lodgepole Pine for Cellulosic Ethanol Production by Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose

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The potentials of deteriorated mountain pine beetle (*Dendroctonus ponderosae*)-killed lodgepole pine (*Pinus contorta*) trees for cellulosic ethanol production were evaluated using the sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) process. The trees were harvested from two sites in the United States Arapaho-Roosevelt National Forest, Colorado. The infestation age of the trees varied from zero to about 8 years. Mild (170 °C) and harsh (180 °C) SPORL pretreatments were conducted. The chemical charges were sulfuric acid of 2.21% and sodium bisulfite of 8% on oven dry wood for the harsh and half of those for the mild pretreatment. The results suggest that beetle-caused mortality enriched glucan content by as much as 3 percentage points (or 7.5%) in wood. The glucan enrichment seems to increase with infestation age. The enriched glucan can be captured after SPORL pretreatment followed by enzymatic hydrolysis. The killed trees are more susceptible to SPORL pretreatment, which enhanced substrate enzymatic digestibility (SED). Enzymatic hydrolysis glucose yields (EHGY) from killed trees were about 5−20% higher than those from their corresponding live trees. Total fermentable sugar productions from dead trees (including a tree laying on the ground) were 4−14% higher than corresponding production from live trees, depending on pretreatment conditions and infestation age. An ethanol yield of 267 L/metric ton of wood or 69% theoretical value was achieved from a tree infested 4 years, 7% higher than the 250 L/metric ton of wood from the corresponding live tree. The results also demonstrated the robustness of SPORL pretreatment for lodgepole pine.

Introduction

Using bioenergy derived from lignocelluloses can help mitigate climate change by the reduction of greenhouse gas emissions and sustainable economic development.[1,2] Forest biomass is a very important lignocellulose feedstock because it is sustainably available in large quantities in many regions of the world, such as Scandinavia, New Zealand, and North and South America. Furthermore, short-rotation intensive culture or tree farming offers an almost unlimited opportunity for forest biomass production.[3,4] Moreover, forest biomass has many advantages over herbaceous biomass, such as high density reducing transportation cost, flexible harvesting time eliminating long-term storage, and near zero ash content, reducing transportation and processing dead load.[5] To promote biodiversity, sustainable and healthy forest and ecosystem management, and to meet local and regional bioenergy needs, woody materials will be a critical part of the biomass supply mix in the future bioeconomy.

Mountain pine beetle (*Dendroctonus ponderosae*) has caused extensive tree mortality in Colorado lodgepole pine (*Pinus contorta*) forests affecting 2.3 million acres from 1996 to 2009. Although a natural disturbance agent of these forests, extensive mortality can also be in conflict with public and private land manager objectives. High tree densities in lodgepole pine forests increase the likelihood of mountain pine beetle infestations or the amount of potential mortality, or both, when large-diameter trees are present (diameter at breast height >20.3 cm). Silvicultural treatments such as thinning from below can reduce stand susceptibility. However, these smaller diameter trees removed during thinning treatments have limited value for structural materials, such as lumber. Cellulosic ethanol production is a feasible pathway for large volume and value-added utilization of lodgepole pine trees infested or killed by mountain pine beetle and for smaller trees removed during thinning operations.

Lodgepole pine (*Pinus contorta*), a softwood, has strong recalcitrance to biochemical conversion to ethanol. Few pretreatment technologies have proven to be effective in removing softwood recalcitrance for satisfactory enzymatic saccharification of cellulose.[5] Organosolv and acid-catalyzed steam explosion pretreatments have been applied to beetle-infested lodgepole pine.[6−8] These studies reported that mountain beetle-killed lodgepole pine was more receptive to organosolv pretreatment than healthy trees. The resultant cellulosic substrate produced from dead trees had a higher substrate enzymatic digestibility (SED, defined as the percentage of glucan in the substrate enzymatically hydrolyzed to glucose) with a slightly lower carbohydrate recovery than that produced from healthy lodgepole pine trees under the same pretreatment conditions.[7] However, the studies did not investigate the effect of infestation age on wood bioconversion. This is critical to the utilization of these trees because of the time delay between onset infestation and harvesting. Furthermore, fermentation was not carried out and therefore cannot provide potential net ethanol energy output from these trees using the organosolv process.

The present study attempts to fill gaps in the literature discussed above on the utilization of mountain beetle-killed

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Table 1. Descriptions of the Six Trees Harvested for the Present Study[a]

site	tree label	GPS coordinates	infestation age and tree conditions	wood chip moisture content (%)
Colorado Front Range	BL	13T0446768 4509250	live	23.5
	BD1	13T0446768 4509250	1 year, dead	15.3
	BD4	13T0447374 4508935	~4 years, dead	11.7
Fraser Experimental Forest	FL	13S0425500 4415649	live	34.4
	FD5	13S0425500 4415649	~5 years, dead	12.7
	FDD	13S0425329 4417171	~8 years, dead, laid on ground	19.1

[a] The first letter represents the site, the second letters, L and D, stand for live and dead, respectively. The number represents the age of infestation.

lodgepole pine for cellulosic ethanol production. The SPORL (sulfite pretreatment to overcome recalcitrance of lignocellulose) process[9] recently developed in our laboratory was used. SPORL is a robust and efficient pretreatment for removing the recalcitrance of woody biomass to enzymatic hydrolysis, including softwood species, with lower energy consumption when compared with acid-catalyzed steam explosion and organosolv processes.[5,9−11] We have achieved an ethanol yield of 276 L/ton of wood with a net energy output of 4.55 GJ/ton of wood from lodgepole pine in a recent study using SPORL.[10] The specific objectives of this study are to determine the time effects of beetle infestation and subsequent fungi decay on tree chemical composition and monosaccharide yield from SPORL process, pretreatment energy consumption for wood mechanical size-reduction and sulfite chemical reactions, and ultimately net ethanol energy output from the trees. The data obtained from this study can provide objective information for developing strategies for utilization of lodgepole pine trees killed by bark beetles for biofuel production.

Experimental Section

Materials. Lodgepole pine trees were harvested from two sites: (a) the Canyon Lakes Ranger District of the Arapaho-Roosevelt National Forest, Colorado, located in the Colorado Front Range (B hereafter), and (b) Fraser Experimental Forest, Front Ranger District of the Arapaho-Roosevelt National Forest, Colorado, located in the west of the continental divide (F hereafter). These two sites were selected for the reasons of different climate and beetle infestation age. The Front Range has a dry climate (east side of the Rocky Mountains in Colorado) and a relatively new insect outbreak of about 4 years, whereas the Fraser Experimental Forest has a wet climate (west side of Rocky Mountains in Colorado) and mountain pine beetle populations have been at outbreak levels for about 9 years at the time of harvesting. Three trees, one living as a control and two mountain pine beetle-killed were harvested at each site. All trees were about 100 years old with a diameter of 20−30 cm at breast height. The times-since-infestation of the dead trees were all different and were determined by the rate of needle and twig loss. One year after infestation, a tree maintains a full crown with bright orange to brown needles; 4 years after infestation, the trees have essentially no needles but large and small twigs remain on the tree.[12] Eight years after infestation, the tree will have fallen by after breaking at the lower bole with sound wood and bark still attached. The specific tree conditions along with GPS (UTM) coordinates of the trees are listed in Table 1. For visual comparison, pictures of the stem cross section and wood chips of selected trees are shown (Figure 1). Moisture content decreased since death (Table 1) except in the wood chips from the deteriorated tree (FDD). Dead trees also contained blue stain caused by a fungus that is introduced into the tree by mountain pine beetles, as observed from the stem cross section and wood chips (Figure 1b). Two blue-staining fungi are known to be associated with mountain pine beetle, *Ophiostoma montium* and *O. clavigerum*. The tree, FDD, Fraser Experimental Forest, was a wind-fall and severely deteriorated as evidenced from the image of tree stem and wood chips (Figure 1c). All trees were cut into logs 1.22 m long after felling. Four logs in addition to the butt log (~0.5 m) were collected from each tree at the Front Range site. The first two logs in addition to the butt log were collected at the Fraser site. The logs were labeled from 0 to 4 from the bottom of the tree (the butt log being 0 and breast height log being 1). The logs were debarked on site during harvesting to eliminate the risk of beetle transport to Wisconsin. Each debarked log was wrapped in a plastic bag to contain any potential beetles during shipping. The debarked logs were then shipped to the U.S. Forest Service, Forest Products Laboratory, Madison, Wisconsin. A 2.5-cm thick wood disk was cut from each log before chipping for chemical composition analysis. Each log was separately chipped, screened, bagged, and stored at −16 °C. Immediately after chipping, the wood chips were screened to remove all particles greater than 38 mm and less than 6 mm in length to ensure smooth operation in disk-milling. The thickness of the accepted chips ranged from 3 to 8 mm (Figure 1).

Celluclast 1.5 L and Novozyme 188 (β-glucosidase) were generously provided by Novozymes, North America (Franklinton, NC). The enzyme activities were 51.4 FPU/mL and 413 CBU/mL for Celluclast 1.5 L and Novozyme 188, respectively, obtained through calibration.[13] Sodium acetate, sulfuric acid, and sodium bisulfite were used as received from Sigma-Aldrich (St. Louis, MO). All other chemicals, including culture media ingredients, were received from Fisher Scientific (Hanover Park, IL). All chemicals were of analytical quality. The Amberlite XAD-4 was also purchased from Sigma-Aldrich (St. Louis, MO). The yeast strain used was *Saccharomyces cerevisiae* D5A, which is available from ATCC culture collections (ATCC 200062). Dr. Bruce Dien of USDA Agricultural Research Service, Peoria, Illinois, graciously provided us with a yeast plate. The stock culture was grown at 30 °C for 2 days on YPD-agar plate containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar. A colony from the plate was then transferred by loop to a 250-mL Erlenmyer flask containing 150 mL of YP media containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. The inoculated culture was grown at 30 °C with agitation at 150 rpm on an orbital shaker.

SPORL Pretreatment. The SPORL experiments were conducted according to the process flow diagram shown in Figure 2 as described elsewhere.[10] Three 1-L stainless steel pressure vessels (manufactured in house) were used to carry out SPORL pretreatment. The three 1-L reactors were mounted inside of a large wood pulping digester as described elsewhere[9] and heated externally using steam. The pulping digester was rotated at the speed of 2 rpm for mixing wood chips with chemicals during pretreatment (free liquor was about 1 L/kg wood based on the amount of collected pretreatment hydrolysate at the end of

Figure 1. Pictures of tree stem and wood chips from selected live and beetle-killed lodgepole pine trees used in this study. (a) BL; (b) BD4; (c) FDD.

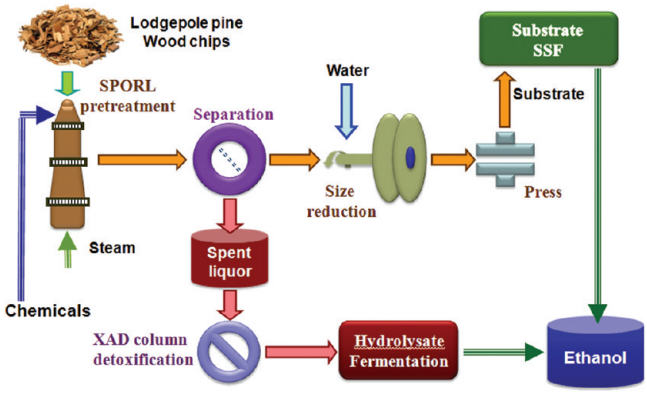

Figure 2. Schematic process flow diagram of the SPORL process with ethanol fermentation.

pretreatment). All wood chips used in pretreatment were from the breast height log (log no. 1). For each batch run, three different wood chip samples from different trees were separately placed in the three reactors and pretreated under the same conditions. To observe the differences in the susceptibility to SPORL pretreatment among different trees, each wood chip sample was pretreated under two different sets of conditions with one being mild and the other relatively harsh. The chemical dosages and pretreatment temperatures for the mild and harsh pretreatments were (Table 2) as follows: charges of sulfuric acid and sodium bisulfite on oven-dry (od) wood of 1.1% and 4% at 170 °C, and 2.2% and 8% at 180 °C, respectively. The pretreatment duration was fixed at 20 min for all the pretreatments conducted, slightly below the 25 min used in our previous study for live lodgepole pine trees.[10] The ratio of pretreatment liquor to od wood chip (L/W) was fixed at 3 (v/w) based on our previous studies.[10,14] Following pretreatment, each 1-L reactor was cooled using tap water while sealed. The residual solids remained as wood chips and were easily separated from the hydrolysate (spent liquor) using a simple screen. The yield of the wood-chip solids was determined from the weight and moisture content of the collected wood chips. This wood-chip solids yield was used to convert the measured energy consumption on pretreated wood chips in the subsequent size-reduction step to that based on an oven-dried untreated wood basis. The pretreatment hydrolysate (spent liquor), which mainly contains hemicellulosic sugars, was poured into a plastic bottle and sealed and stored at 4 °C until used for analysis and fermentation. Losses of volatiles should be minimal from the collected hydrolysate.

Size-Reduction of Pretreated Wood Chips. The approach of postchemical pretreatment disk milling was applied to reduce energy consumption for wood size-reduction.[5,14] The pretreated wood chips were directly transferred to a laboratory 12-in. disk mill (Andritz Sprout-Bauer Atmospheric Refiner, Springfield, OH) for size-reduction under atmospheric conditions (Figure 2). The two disk-plates have a pattern of D2-B505. All disk-milling runs were conducted at 2570 rpm with a disk plate gap of 1.0 mm and solids-loading of 10%. The milling solids-loading is defined as the percentage of pretreated wood chip (od basis) in the total feed into the mill, where the total feed includes chips "as is" and added water. The substrate (size-reduced solids) was not separately washed and was directly dewatered through pressing using a canvas bag to a solids content of about 30%. The yield of solid (substrate) in the form of fibers or fiber bundles was then determined from the weight and moisture content of the collected substrate. The moisture content was determined gravimetrically by drying a sample of the collected solids in an oven at 105 °C overnight. This solid substrate yield

Table 2. SPORL Pretreatment Conditions Used To Evaluate the Utility of Beetle-Killed Lodgepole Pine for Ethanol Production[a]

	sample label	T (°C)	acid charge (wt % wood)	bisulfite charge (wt % wood)	liquor initial pH	final liquor pH (average)
mild	BL-7-4, BD1-7-4, BD4-7-4; FL-7-4, FD5-7-4, FDD-7-4	170	1.10	4	2.24	1.80
harsh	BL-8-8, BD1-8-8, BD4-8-8; FL-8-8, FD5-8-8, FDD-8-8	180	2.21	8	2.11	1.58

[a] Pretreatment duration was 20 min and liquor-to-wood ratio (L/W) was 3 (v/w) for all the runs. The numbers in the sample labels represent temperature and percent of bisulfite charge for the first and second number, respectively.

was used to convert the measured substrate glucan content and enzymatic hydrolysis glucose yield (EHGY) from substrate base to untreated wood base for process mass balance analysis.

The electrical energy consumption for size-reduction by disk-milling was determined using a digital load monitor system (Ohio Semitronics, Inc., Hilliard, OH, model DLM-33-480-1PR) as previously described.[15] The determined energy divided by the od mass of pretreated wood chips fed into the mill gives energy consumption in Wh/kg of od fed chips, which was then multiplied by the yield of wood-chip solids after SPORL pretreatment to yield energy consumption for size-reduction, in Wh/kg of od untreated wood.

Wood Chemical Composition Determination and Analytical Methods. Wood chemical compositions were analyzed to study the effect of time-since-beetle-caused mortality on wood chemical composition. Initially, 2.5 cm thick wood disks were cut from the topmost portion of each log. These disks were then oven-dried at 105 °C overnight to prevent any local fungi infestation. Next, a 90° sector was band-sawed from each of the disks, such that the sector encompassed the area of the disk with the longest pith-to-cambium radius. These sectors were then manually broken into ca. $2.5 \times 2.5 \times 1.0$ cm^3 blocks with a wood chisel and hammer. The resulting blocks were then ground in a Wiley mill (model no. 2, Arthur Thomas Co, Philedelphia, PA) using a 4-mesh outlet screen (~5 mm). This was followed by a second pass using a 20-mesh (~1 mm) screen. The resulting materials were sent to the Analytical Chemistry and Microscopy Laboratory (ACML) of the U.S. Forest Service, Forest Products Laboratory for analysis. The samples were first hydrolyzed using sulfuric acid in two stages. The hydrolysis conditions were an acid concentration of 72% (v/v) at 30 °C and 3.6% (v/v) at 120 °C for the first and second stages, respectively. The hydrolysis duration time was 1 h for both stages. The hydrolysate was then analyzed for carbohydrates using an improved high-performance anion exchange chromatographic method using pulsed amperometric detection (HPAEC-PAD).[16] The Klason lignin content was measured gravimetrically after washing and drying the solid residue from the acid hydrolysis. The average results from four replicate samples from each breast height disk (log 1) were compared to study the effect of infestation age on wood chemical composition among the different trees. The standard deviations were calculated from the four replicate runs as measurement errors. The average data of duplicate samples from each of the remaining wood disks (logs 0, 2−4) was used for tree comparison to study the effect of juvenile wood on wood chemical composition relative to tree height.

The same Wiley milling, hydrolysis, and anion chromatographic methods were applied to pretreated solid substrates to determine their chemical compositions. The saccharides in the pretreatment hydrolystaes (spent liquors) were analyzed using a Dionex HPLC system (ICS-3000) equipped with integrated amperometric detector and Carbopac PA1 guard and analytical columns at 20 °C. Eluent was provided at a rate of 0.7 mL/min, according to the following gradient: 0 to 25 min, 100% water; 25.1 to 35 min, 30% water and 70% 0.1 M NaOH; 35.1 to 40 min, 100% water. To provide a stable baseline and detector sensitivity, 0.5 M NaOH at a rate of 0.3 mL/min was used as postcolumn eluent. Fermentation inhibitors generated in pretreatment including acetic acid, formic acid, furfural, levulinic acid, and 5-hydroxylmethylfurural (HMF) were analyzed using the same system with a Supelcogel C-610H column at 30 °C and UV detector at 210 nm. Eluent was 0.1% phosphoric acid at a rate of 0.7 mL/min. To determine the oligomeric saccharides, the pretreatment hydrolysates were further hydrolyzed by adding an equal volume amount of sulfuric acid of 6% (v/v) to make samples with sulfuric acid concentration of about 3% (v/v). The acid hydrolysis was conducted in an autoclave at 120 °C for 60 min. All hydrolysate data reported were averages of triplicate measurements. The measurement errors were the standard deviations of the triplicate analyses. For fast analysis, glucose in the enzymatic hydrolysate was measured in duplicate using a commercial glucose analyzer (YSI 2700S, YSI Inc., Yellow Springs, OH).

Ethanol analyses in the fermentation broths of cellulosic substrates and pretreatment hydrolysates were carried out using a gas chromatograph (model 7890, Agilent Technologies, Palo Alto, CA) through direct sample injection using an external standard for calibration. The chromatograph is equipped with a flame ionization detector (FID) and Agilent DB-Wax column of 30 m with an ID 0.32 mm. A universal guard column was used to reduce column contamination.

Enzymatic Hydrolysis. Separate enzymatic hydrolysis experiments of the pretreated substrates were conducted to measure the enzymatic hydrolysis glucose yield (EHGY) in terms of kg/ton of untreated wood. Enzymatic hydrolysis was conducted using commercial enzymes at 2% substrate solids (w/v) in 50-mL of sodium acetate buffer (pH 4.8, concentration 50 mM) on a shaker/incubator (Thermo Fisher Scientific, model 4450, Waltham, MA) set at 50 °C and 200 rpm. An enzyme mixture of Celluclast 1.5 L cellulase (15 FPU/g substrate) and Novozyme 188 β-glucosidase (22.5 CBU/g substrate) was used for hydrolysis. Hydrolysate was sampled periodically for glucose concentration. Each data point is the average of replicates.

Pretreatment Hydrolysate Detoxification and Fermentation. Pretreatment hydrolysates (spent liquor) were preconditioned for fermentation using XAD-4 to absorb furan inhibitors as described elsewhere.[10] Amberlite XAD-4 (15 g, Rohm and Haas, Philadelphia, PA) was loaded into a 1.5×15 cm^2 glass column and washed with 3× volume of water. The SPORL hydrolysate (50 mL/batch) was pumped onto the column at ambient temperature and the elution was only collected once the hydrolysate began to exit the column as judged by color. The XAD-4 was replaced for each batch of hydrolysate. Following absorption, the hydrolysate was adjusted with $Ca(OH)_2$ to pH 5 and filter sterilized by passing through a 0.22 μm filter, which may also remove some solids, such as $CaSO_4$. The ethanol fermentations were performed in 250 mL Erlenmeyer flasks with a working volume of 50 mL. The solution was inoculated with *S. cerevisiae* D5A to an initial cell mass

Table 3. Effect of Beetle Infestation Age on Wood Chemical Compositions (wt %) of Lodgepole Pine. The Last Number (1) in Sample Label Denotes Breast Height Log

wood log	K lignin	arabinan	galactan	glucan	xylan	mannan	glucan + mannan	total
BL-1	29.1 ± 0.1	1.8 ± 0.1	3.4 ± 0.3	39.8 ± 0.3	6.8 ± 0.5	10.1 ± 0.6	49.9	93.4
BD1-1	29.1 ± 0.1	1.7 ± 0.0	3.3 ± 0.1	40.3 ± 0.2	5.3 ± 0.1	11.6 ± 0.3	51.9	91.5
BD4-1	28.6 ± 0.2	1.7 ± 0.2	2.9 ± 0.4	41.9 ± 0.6	5.5 ± 0.5	11.7 ± 0.3	53.6	93.8
FL-1	29.2 ± 0.2	2.1 ± 0.1	4.2 ± 0.1	39.1 ± 0.6	6.0 ± 0.3	10.0 ± 0.6	49.1	93.5
FD5-1	28.6 ± 0.3	1.5 ± 0.1	2.8 ± 0.2	41.7 ± 0.6	5.9 ± 0.4	11.3 ± 0.6	53.0	93.4
FDD-1	28.2 ± 0.3	1.1 ± 0.1	2.4 ± 0.1	42.0 ± 0.0	4.6 ± 0.1	9.5 ± 0.1	51.5	88.0
average standard deviation	0.2	0.1	0.2	0.5	0.3	0.5		

concentration 1.0 g/L(wet basis). The culture was incubated at 30 °C and agitated at 100 rpm for 72 h using a shaker/incubator without adding any nutrients. Samples taken every 24 h were stored at −20 °C until analyzed for sugars and ethanol.

Quasi-simultaneous Enzymatic Saccharification and Fermentation (SSF). SSFs were carried out in 250 mL Erlenmeyer flasks using a shaker/incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) set at 35 °C and 90 rpm with about 8% substrate (water insoluble, 4 g of substrate in 50 mL of enzyme/buffer solution of pH 4.8). The enzyme loading of Celluclast 1.5 L was 20 FPU/g substrate and Novozyme 188 (β-glucosidase) of 30 CBU/g substrate, a little higher than that used for simple enzymatic hydrolysis considering the increased solids loading of 8%. Liquefaction of the solid substrates was initiated in about 2 h at 50 °C and 200 rpm before adding yeast. The opening end of the flasks was then wrapped with aluminum foil to avoid ethanol leak. No additional nutrients were added during fermentation. The initial cell concentration for SSF was 2 g/L (wet base). Samples of the fermentation broth were taken every 24 h for ethanol analysis. All fermentation experiments were ended after 96 h. Reported results are the average of duplicates.

Results and Discussions

Effects of Time-since-Death on Wood Chemical Composition. The carbohydrate content of wood directly affects its utility for biochemical conversion to ethanol. Table 3 lists the wood chemical compositions measured from the disk of each breast height log (log 1, the last number in sample label in Table 3) of the six trees harvested. When comparing carbohydrate data of the beetle-killed trees with the corresponding live trees, the effect of glucan enrichment by fungi decay is apparent. The glucan content was increased by as much as 3 percentage points (or 7.5%) for the two sets of trees harvested from the Front Range (B), and Fraser Experimental forest (F), respectively. The data also indicate that the glucan enrichment is caused by the reduction in arabinan, galactan, and xylan. The average (of six trees) standard deviations of glucan (0.5%), arabinan (0.1%), galactan (0.2%), and xylan (0.3%) are much smaller than the differences between the live (L) and dead (D) trees (Table 3). Furthermore, the data suggest that the enrichment of glucan and the reductions in pentosan increase with infestation age. ANOVA analysis indicated that at 95% confidence level the differences in glucan content are statistically significant between the BL/BD1 and BD4, FL and FD5/FDD. The data also indicate the enrichment of mannan by fungi decay except for the deteriorated tree (FDD). The differences in mannan content between the live (L) and dead (D) trees are larger than the measurement error (Table 3). ANOVA analysis indicated that at 95% confidence level the mannan enrichment is statistically significant between BL and BD1/BD4, FL and FD5. The lignin content of the beetle-killed wood was reduced slightly (Table 3). These observations agree with those reported previously.[7] Chemical composition analyses of different logs from the same tree found no statistical differences in lignin, glucan, and hemicellulose contents along tree height direction, except when juvenile wood becomes a factor at the very top of the tree. This suggests that the glucan enrichment by fungi decay observed at the breast height is true for the entire tree.

Effects of Fungi Decay on Saccharides Recovery from SPORL Pretreatment. Saccharide recovery not only can be used to access the performance of a pretreatment process, but also to evaluate the susceptibility of the biomass feedstock for biochemical conversion. All six wood chip samples produced from the breast height log (Log 1, Table 3) of the six trees were subjected to SPORL pretreatment under two sets of conditions listed in Table 2. The yields of various components from solid substrates and pretreatment hydrolysates were separately determined. A minimal glucan loss of about 10% or less, from the solid substrate, was found for all the wood chip samples evaluated under both the mild and harsh pretreatment conditions (Table 4). The differences in glucan loss through SPORL pretreatments among live and infested trees are within the measurement errors (contributed by the uncertainties in both carbohydrate analyses and substrate yields measurements). Under the harsh SPORL pretreatment, glucan recoveries from solid substrates from beetle-infested trees (including the deteriorated tree, FDD) are higher (about 4−8%) than are those from the corresponding live trees (Table 4). This can be attributed to the higher glucan content of the beetle-infested trees. The older is the infestation age of the tree, the higher is the glucan recovery achieved, with the exception for the deteriorated tree (FDD) under the harsh pretreatment conditions (Table 4).

The removal of hemicelluloses is not affected by beetle infestation (Table 4). Although about 80% or more of the xylan and mannan were removed from wood by SPORL pretreatments, generally less than half were converted to monomeric sugars (Table 4). To account for the differences in hemicelluloses recoveries in the hydrolysates, the oligosaccharides in the pretreatment hydrolysates were determined by subtracting the monosaccharides in the original spent liquor (hydrolysate) from those measured in the sulfuric acid hydrolyzed spent liquor (hydrolysates). The results suggest that about 26−36% of the glucan, 22−28% of the mannan, and 8−20% of the xylan remained as oligomers in the spent liquor under the mild SPORL pretreatment. The percentages dropped to about 10−20, 7−10, and, 5−13% for glucan, mannan, and xylan, respectively, under the harsh SPORL pretreatment. Although fungi decay did not significantly affect glucan loss from solid or hemicellulose removal, the results indicate that infestation increased monosaccharide glucose and mannose recoveries from pretreatment hydrolysate (Table 4). The exception is for the mannose from the deteriorated tree (FDD) under the harsh SPORL pretreatment. Beetle infestation reduced xylose recoveries from pretreatment spent liquor owing to the reduced xylan content.

Table 4. Yields of Key Components in the Recovered Solids and Pretreatment Hydrolysate Based on 1000 kg of od Wood after SPORL Pretreatments under Two Sets of Chemical Applications. Pretreatment Duration and Liquor to Wood Ratio (L/W) Were Fixed at 20 min and 3, Respectively. All Data Are in kg

	solid substrates					pretreatment hydrolysates									
sample	glucan (% loss)	xylan (% loss)	mannan (% loss)	K lignin	solids yields[a]	glucose as glucan	oligo. glucan	xylose as xylan	oligo. xylan	mannose as mannan	oligo. mannan	K lignin[b]	furfural as pentosan	HMF as hexosan	yield[c]	acetic acid	total yield
BL-7-4	363.9 (8.6)	11.7 (82.9)	7.7 (92.3)	268.1	664.8	14.7 ± 0.47	6.5	33.0 ± 1.01	4.6	43.5 ± 1.07	17.6	22.9	4.4 ± 0.21	2.6 ± 0.22	149.8	10.3 ± 1.66	824.9
BD1-7-4	366.2 (9.2)	10.2 (80.7)	8.1 (93.0)	259.2	639.9	15.1 ± 0.56	6.9	27.6 ± 1.33	5.0	48.7 ± 1.83	18.7	31.8	3.3 ± 0.26	2.4 ± 0.25	159.4	10.1 ± 0.33	809.4
BD4-7-4	377.4 (9.9)	10.8 (80.4)	5.4 (95.4)	264.9	650.4	19.0 ± 0.71	6.8	27.6 ± 1.56	5.6	54.4 ± 1.46	19.3	21.1	3.2 ± 0.27	2.6 ± 0.15	159.6	11.8 ± 0.25	821.8
FL-7-4	371.5 (5.0)	9.9 (83.5)	11.1 (88.9)	300.1	693.1	12.8 ± 0.47	6.5	30.3 ± 1.65	4.5	41.7 ± 1.43	14.1	N.D.	4.0 ± 0.27	2.4 ± 0.20	116.3	7.8 ± 0.20	817.2
FD5-7-4	383.9 (8.0)	12.9 (78.1)	14.7 (87.0)	241.9	667.6	15.2 ± 0.78	5.5	29.6 ± 1.61	2.6	48.7 ± 2.18	13.4	44.1	3.4 ± 0.12	2.4 ± 0.24	164.8	9.4 ± 1.9	841.8
FDD-7-4	391.2 (6.9)	10.8 (76.3)	12.5 (86.9)	253.5	682.6	14.9 ± 0.53	8.4	21.8 ± 0.98	5.1	39.3 ± 1.17	15.2	28.5	3.1 ± 0.10	2.4 ± 0.06	138.6	6.4 ± 0.03	827.6
BL-8-8	362.7 (8.9)	7.4 (89.2)	4.4 (95.6)	249.8	619.0	18.8 ± 0.65	3.0	27.6 ± 0.76	1.8	45.9 ± 1.75	4.0	41.2	8.4 ± 0.07	5.6 ± 0.14	171.2	11.2 ± 0.07	801.4
BD1-8-8	376.6 (6.6)	7.3 (86.1)	4.4 (96.2)	213.4	614.0	20.6 ± 0.61	3.3	22.3 ± 0.81	1.1	52.7 ± 1.17	4.2	77.6	6.1 ± 0.08	5.7 ± 0.13	211.6	12.6 ± 0.61	838.2
BD4-8-8	381.6 (8.9)	9.5 (82.8)	4.1 (96.5)	218.4	621.8	23.0 ± 0.23	2.5	22.6 ± 0.32	2.4	58.8 ± 1.01	4.5	67.6	5.4 ± 0.19	5.2 ± 0.06	205.8	12.5 ± 0.13	840.1
FL-8-8	351.1 (10.2)	8.6 (85.7)	9.1 (85.7)	241.3	634.2	16.2 ± 0.15	3.5	23.9 ± 0.43	2.3	42.8 ± 0.24	3.6	50.7	6.0 ± 0.51	4.3 ± 0.07	167.8	8.5 ± 0.04	810.5
FD5−8-8	383.3 (8.2)	11.2 (81.0)	14.9 (81.0)	219.7	642.6	18.2 ± 0.32	4.6	23.0 ± 0.97	1.7	51.7 ± 0.87	5.5	66.3	5.3 ± 0.15	4.4 ± 0.18	195.8	9.3 ± 0.07	847.7
FDD-8-8	379.5 (9.6)	7.0 (84.7)	7.2 (84.7)	207.4	629.6	17.7 ± 0.46	3.3	17.0 ± 0.38	2.6	40.8 ± 0.42	4.6	74.6	4.6 ± 0.16	4.2 ± 0.10	188.1	7.7 ± 0.47	825.4

[a] As measured after disk-milling. [b] Based on balance of lignin. [c] Sum of listed pretreatment hydrolysate components (acetic acid excluded).

Figure 3. Effect of beetle infestation on time-dependent substrate enzymatic digestibility of the trees harvested from Front Range (B).

Effects of Fungi Decay on SPORL-Pretreated Substrate Enzymatic Digestibility (SED). Separate enzymatic hydrolyses of substrates produced from two SPORL pretreatments on the six wood chip samples were conducted to assess the effect of beetle infestation on wood susceptibility to SPORL pretreatment. It was found that beetle infestation increased substrate enzymatic digestibility (SED) under both SPORL pretreatments for the trees from Front Range (Figure 3). Furthermore, SED increased with the infestation age under a mild SPORL pretreatment, that is, SED of BD4 is higher than that of BD1. Under a harsh pretreatment, the SED of BD4 was slightly lower than that of BD1 while the SEDs of both BD1 and BD 4 are substantially (>7 percentage points) higher than the SED of BL (Figure 3). The maximal SED of BD4 of 92% was achieved in 48-h hydrolysis. Similar results were obtained for the trees harvested from the Fraser Experimental Forest (F) but with varied degrees because of the difference in tree infestation age; for example, the SED of the deteriorated tree (FDD) is substantially higher (8 percentage points) than the SED of the live tree (FL) under mild pretreatment conditions. These results suggest that beetle-infested trees are more susceptible to SPORL pretreatment to improve cellulose saccharification and agree with that reported by Pan et al.[7] using organosolv pretreatment.

Effects of Beetle Infestation on Fermentable Sugar Yield, Sugar Production Energy Efficiency, And Inhibitor Formation. The total fermentable sugar yields, fermentation-inhibitor formations, and sugar-production energy efficiencies were determined for the six wood samples studied to provide a better understanding of the utility of beetle-infested trees for cellulosic ethanol production. The total sugar yield was the sum of enzymatic hydrolysis glucose yield (EHGY), defined as the amount (kg) of glucose produced from one metric ton of od wood, and the yields of glucose, xylose, and mannose from the pretreatment hydrolysates (Table 4). The results indicate that beetle-infested trees not only showed greater substrate enzymatic digestibility (SED) as discussed in the previous section, they also produced 5−20% higher EHGY (Table 5) than the corresponding live trees. As a result, the total fermentable sugar yields were higher from the beetle-infested trees than the corresponding live trees (Table 5). The highest sugar recovery was 490 kg/ton of wood (or theoretical sugar yield of 75% based on wood glucan, xylan, and mannan contents) from the tree infested 4 years (BD4) from the Front Range, and 452 kg/ton of wood (theoretical yield of 69%) from the tree infested for 5 years (FD5) from the Fraser Experimental Forest.

The energy consumptions for wood size-reductions based on untreated wood were contributed by both the energy consumption in milling of pretreated wood chips and wood chip solids

Table 5. Monomeric Sugar Recoveries and Pretreatment Energy Efficiencies under Two SPORL Pretreatments

sample label	wood milling energy[a]	total pretreatment energy[a]	EHGY @72 h[b]	hydrolysate fermentable sugar[b]	total fermentable sugar[c]	$\eta_{pretreatment}$[d]
BL-7-4	1.14	2.57	205.5	101.3	306.9/48.6	0.120
BD1-7-4	1.07	2.49	225.2	101.6	326.8/51.3	0.131
BD4-7-4	1.07	2.50	245.9	112.2	358.0/54.4	0.143
FL-7-4	1.40	2.82	212.4	94.2	306.7/50.0	0.109
FD5-7-4	1.02	2.44	213.3	103.9	317.2/48.4	0.130
FDD-7-4	1.27	2.70	257.7	84.5	342.1/54.8	0.127
BL-8-8	0.59	2.10	326.5	102.6	429.1/68.0	0.205
BD1-8-8	0.61	2.12	377.6	106.2	483.8/75.9	0.228
BD4-8-8	0.65	2.34	374.1	115.9	490.1/74.5	0.227
FL-8-8	0.68	2.19	321.5	92.1	413.7/67.4	0.189
FD5-8-8	0.73	2.24	348.6	103.2	451.8/68.9	0.202
FDD-8-8	0.46	1.97	338.4	83.8	422.2/67.6	0.214

[a] In GJ/metric ton of untreated wood. [b] In kg/od metric ton of wood. Data for hydrolysate were measured before detoxification. [c] The first number is in kg/od metric ton of wood and the second number is wt% of theoretical, based on glucan, xylan, and mannan content in wood (Table 3). [d] In kg/MJ.

Table 6. Comparison of Ethanol Yield and Ethanol Production Energy Efficiency between a Live (BL) and a Beetle-Infested Tree (BD4)

sample label	substrate glucose[a]	SSF ethanol[b]	SSF fermentation efficiency[c]	hydrolysate glucose + mannose[a]	hydrolysate ethanol[b]	hydrolysate fermentation efficiency[c]	total ethanol yield[d]	ethanol energy[e]	wood milling energy[e]	total energy input[e]	η_{energy} (%)
BL-8-8	403.0	239.0/24.4	91.6	83.3/27.8	11.2/3.0	20.8	250.2/69.7	5.86	0.24	1.75	234.9
BD4-8-8	424.0	254.7/25.9	92.8	95.1/31.7	11.8/3.1	19.2	266.5/69.1	6.23	0.16	1.67	272.8

[a] The first number is in kg/od metric ton of wood based on measured glucan contents and substrate yields. Data for hydrolysate were after detoxification. The second number after the slash is measured concentration in g/L. [b] The first number is in L/od metric ton of wood. The second number is measured concentration in the fermentation broth in g/L. [c] Percentage of theoretical ethanol (0.511 g ethanol/g hexose) yield from substrate glucose or detoxified hydrolysate glucose and mannose. [d] The first number is in L/od metric ton of wood and the second number is wt% of theoretical yield based on glucan and mannan content in wood (Table 3). [e] In GJ/ton of untreated wood.

yield from pretreatment. The results show some mixed small variations (Table 5) contributed by both the true differences among different samples and the measurement uncertainties in the measured rotor power and wood chips solids yields. For example, the results show slight reduction in energy consumption for milling beetle-infested trees after the mild SPORL pretreatment (Table 5). However, except for the deteriorated tree, the data show slight increases when the harsh SPORL pretreatment was applied. We estimated the total pretreatment energy consumption by assuming a constant energy consumption for wood chipping of 50 KWh/ton of wood and a 50% thermal energy recovery in SPORL thermochemical pretreatment, based on pulp and paper industrial practice. The thermal energy consumption for SPORL thermochemical pretreatment is simply the enthalpy increase of the wood chip suspension. According to Zhu and Pan,[5] pretreatment energy efficiency is defined as

$$\eta_{pretreatment} = \frac{\text{total monomeric sugar yield}}{\text{total energy consumption in pretreatment}} \quad (1)$$

The results clearly show that beetle infestation increased pretreatment energy efficiency (Table 5) by about 10% or more for both of the mild and harsh SPORL pretreatments conducted. Fermentation inhibitors can interfere with fermentation of pretreatment hydrolysate. The results indicate that furfural formations were slightly lower from beetle-infested trees than their corresponding live trees (Table 4), probably due to the low pentosan content of the infested trees (Table 3). HMF formation was not affected by beetle infestation (Table 4).

Ethanol Yields and Process Energy Efficiency. Simultaneous enzymatic saccharification and fermentation (SSF) of solid substrates and fermentation of pretreatment hydrolysates were conducted to further verify the benefits of fungi decay as discussed previously. The yeast strain D5A used can only ferment hexose, that is, mainly glucose and mannose. The fermentation efficiency and ethanol yield are therefore calculated on the basis of glucose and mannose content in this study. The solid substrates and pretreatment hydrolysates were produced from separate SPORL pretreatments, but under the same harsh conditions (Table 2), and applied to two Front Range wood chip samples from BL (the live tree) and BD4 (beetle-killed for 4 years). The results (Table 6) clearly show that 7% more total ethanol was produced from BD4 (267 L/metric ton of wood) than that from BL (250 L/metric ton of wood). The SSF fermentation efficiencies for both of the substrates were over 90%, suggesting the robustness of SPORL pretreatment in removing recalcitrance of lodgepole pine (a softwood). The results also indicate that the higher total ethanol yield of 267 L/ton of wood from BD4 than the 250 L/ton of wood from BL is from SSF of the solid cellulosic substrates (255 vs 239 L/metric ton of wood) and not from fermentation of the pretreatment hydrolysate (primarily hemicellulose sugar stream). This proves that glucan enrichment by beetle infestation and subsequent fungi decay produced tangible gain in ethanol yield. The ethanol productions from the detoxified pretreatment hydrolysates of these two wood chip samples were about the same at 11 L/ton of wood with fermentation efficiency of only about 20%, and were lower than that reported in our previous study.[10] This can be attributed to two factors: (1) nutrients were not added in fermentation in this study and (2) the hydrolysates obtained in the present study have lower sugar concentrations due to a shorter pretreatment time of 20 min. The initial concentrations of inhibitors, acetic acid, furfural, and HMF in the pretreatment hydrolysates were 4.02, 2.67, and 3.11 g/L for BL and 8.93, 2.34, and 2.75 g/L for BD4, respectively. Our previous study found that the *S. cerevisiae* D5A was incapable of fermenting SPORL hydrolysates directly without detoxification despite that lower amounts of furan were produced when compared with that of the dilute acid pretreatment.[10] Our recent study indicates that direct fermentation of the combined SPORL enzymatic hydrolysate with undetoxified SPORL hydrolysate

can produce excellent fermentation efficiency,[17] suggesting that fermentation of the combined stream is more efficient to eliminate detoxification. It should be pointed out that despite using the same prescribed SPORL pretreatment conditions, the 90% SSF fermentation efficiency was slightly higher than that of the SED shown in Figure 3. This could be explained by two reasons: (1) The enzyme loading was 20 FPU/g substrate in SSF higher than the 15 FPU/g substrate used to produce the SED data in Figure 3. (2) It is possible that the SPORL pretreatment that produced the substrate for SSF was actually more severe than the pretreatment that produced the results in Figure 3 and Tables 4 and 5. This is likely due to the variations of laboratory steam pressure. This can be verified from the lower disk-milling size-reduction energy consumption (Table 6) compared with the corresponding values reported in Table 5. A more severe pretreatment produced a solids substrate that requires less energy in mechanical milling due to more severe degradation of wood chips.

The ethanol production energy efficiency can be defined as[10]

$$\eta_{\text{energy}} = \frac{\text{net energy output}}{\text{total energy input}} \quad (2)$$

The net ethanol yields (Table 6) resulted in process ethanol energy efficiency of 235 and 273 for the live (BL) and beetle-infested tree (BD4), respectively. Only energy consumed for pretreatment was used. Mixing energy for SSF was ignored as it was conducted in a shaking bed at low consistency of 8%.

Conclusions

This study evaluated the effects of mountain beetle infestation and subsequent fungi decay on lodgepole pine for cellulosic ethanol production. It was found that fungi decay enriches glucan content in the beetle-killed trees, including the deteriorated tree up to about 3 percentage points or ~7.5%. The enrichment seems to increase with infestation age up to about 5 years. The enriched glucan was retained on the cellulose substrate after SPORL pretreatment. Results from the pretreatment and subsequent enzymatic hydrolysis of the pretreated solid substrates indicate that beetle infestation increased wood susceptibility to SPORL pretreatment and substrate digestibility (SED). Glucan recoveries from the infested trees were 4−8% higher from the infested and decayed trees (including the deteriorated tree FDD) than that from the corresponding live trees for the two SPORL pretreatments conducted. Beetle infestation did not significantly affect hemicellulose removal in pretreatment, but slightly increased the recoveries of glucose and mannose from the pretreatment hydrolysate. Results from enzymatic hydrolysis of the pretreated solid substrates indicate that beetle infestation and subsequent fungi decay increased wood susceptibility to SPORL pretreatment and increased substrate digestibility (SED). Enzymatic hydrolysis glucose yield (EHGY) from infested trees were about 5−20% higher than that from the corresponding live trees due to combined effects of enhanced SED and increased substrate solid yield. Total fermentable sugar production from infested trees (including the deteriorated tree FDD) was about 4−14% higher than that from corresponding live trees, depending on pretreatment conditions and infestation age. Fermentation results further prove the tangible gain of about 7% in ethanol yield from a dead tree. Ethanol yields of 267 and 250 L/metric ton of wood or about 69% theoretical value from a dead (BD4) and a live (BL) tree, respectively, were achieved without process optimization, suggesting the robustness of SPORL pretreatment for ethanol production from woody biomass, including softwood species. The results presented in this study can provide information for landowners and policy makers in determining the utility of beetle-killed stands when making decisions regarding high-value utilization of infested trees, sustainable feedstock production and biodiversity, and energy production and its environmental impact, for healthy forest management.

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