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High titer ethanol production from SPORL-pretreated lodgepole pine by simultaneous enzymatic saccharification and combined fermentation ☆

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HIGHLIGHTS

- ▶ High titer of 47 g/L and high yield of 285 L/tonne wood ethanol production from lodgepole pine.
- ▶ Simultaneous enzymatic saccharification of pretreated solids and combined fermentation with pretreatment spent liquor.
- ▶ Fed-batch of unwashed solids in fermentation to manage inhibitor and to achieve high ethanol titer.

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ABSTRACT

Lodgepole wood chips were pretreated by sulfite pretreatment to overcome recalcitrance of lignocelluloses (SPORL) at 25% solids loading and 180 °C for 20 min with sulfuric acid and sodium bisulfite charges of 2.2 and 8 wt/wt% on an oven-dry wood basis, respectively. The pretreated wood chips were disk-milled with pretreatment spent liquor and water, and the solid fraction was separated from the liquor stream. The liquor was neutralized and concentrated through vacuum evaporation. Quasi-simultaneous enzymatic saccharification of the cellulosic solids and combined fermentation with the concentrated liquor was conducted at up to 20% total solids loading. Fed-batching of the solids facilitated liquefaction and saccharification, as well as managing instantaneous inhibitor concentrations. At a commercial cellulase (CTec2) loading of only 9 FPU or 0.06 mL/g untreated wood, a maximum ethanol titer of 47.4 g/L was achieved, resulting in a calculated yield of 285 L/tonne of wood using *Saccharomyces cerevisiae* YRH400 at 35 °C and pH 5.5.

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1. Introduction

Substituting biofuel from lignocellulosic biomass for petroleum based liquid fuel can help to mitigate climate change and sustainable economic development; however, barriers such as robust pretreatment, high solids enzymatic saccharification, efficient fermentation, and low energy separation need to be surmounted (Lynd et al., 2008; Zhu and Pan, 2010; Zhu and Zhuang, 2012). Despite significant progress in the last two decades, integration of biorefinery process technologies remains a challenge, partly because integration requires a coordinated effort, and optimization needs a systematic approach to examine overall process performance. For example, increasing pretreatment severity often results in high total sugar yield; however, acid pretreatment will also increase the production of fermentation inhibitors in the hemicellulosic sugar stream (Larsson et al., 1999; Zhu et al., 2012). Washing pretreated solids can reduce enzyme inhibition by lignin and improve the efficiency of cellulose saccharification (Nagle et al., 2002; Tengborg et al., 2001), but washing consumes a significant amount of water (Liu and Zhu, 2010). While high solids processing can increase biofuel titer favorable to downstream separation, it requires high enzyme loading and energy costs for mixing (Liu et al., 2010; Zhang et al., 2010a).

Several integrated biorefinery studies have been reported that presented results from unprocessed biomass to biofuel production; however, most of these have been conducted at low solids levels with a final bioethanol concentration of less than 40 g/L (Hoyer et al., 2010; Jin et al., 2012; Monavari et al., 2010; Nieves et al., 2011; Tian et al., 2010; Zhu et al., 2010). Low solids processes can avoid some of the difficulties encountered when attempting enzymatic saccharification with high solids loadings. Likewise fermentation of dilute hydrolysate can avoid high concentrations of

☆ This work was conducted at the USDA Forest Service, Forest Products Laboratory while Lan was a visiting student from South China University of Technology and on official government time of Zhu, Gleisner, Dien, and Hector.

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microbial inhibitors; however, processes using high solids loading could have economic advantages. Fed-batch enzymatic saccharification with simultaneous fermentation (SSF) has the potential to address the difficulty of high solids liquefaction for effective cellulose saccharification as demonstrated using pretreated corncobs (Liu et al., 2010; Zhang et al., 2010b), a lignocellulosic feedstock with relatively low recalcitrance. Fed-batch SSF can also reduce fermentation inhibition without detoxification. For example, saccharification and fermentation of spruce pretreated with SO_2-catalyzed steam explosion at a moderate (water insoluble) solids loading of 14% produced good ethanol yield without detoxification (Hoyer et al., 2010). The effectiveness of the fed-batch strategy still needs to be demonstrated for ethanol production at high titers (>40 g/L) using a very recalcitrant feedstock such as woody biomass.

The objective of the present study is focused on achieving high titer (>40 g/L) ethanol production from strongly recalcitrant softwood using quasi-simultaneous enzymatic saccharification and combined fermentation following SPORL pretreatment of lodgepole pine. The SPORL pretreatment resulted in a solid cellulosic fraction and non-detoxified pretreatment hydrolysate (spent liquor). The study described herein established baseline conditions for cellulase dosing to achieve efficient saccharification of unwashed whole hydrolysate and co-fermentation of enzymatically saccharified solids at high loading without detoxification. This study is built on the success achieved in cellulosic ethanol production from very recalcitrant softwood using SPORL (Tian et al., 2010; Wang et al., 2012; Zhu et al., 2009, 2010).

2. Methods

2.1. Materials

Lodgepole pine wood chips were produced at the Forest Products Laboratory, Madison, Wisconsin, from a mountain pine beetle-killed tree (abbreviated BD4) harvested from the Canyon Lakes Ranger District of the Arapaho-Roosevelt National Forest, Colorado, USA. Detailed information about the tree, harvesting, and transportation have been described previously (Luo et al., 2010). The bioconversion of beetle-killed lodgepole pine was found slightly easier than that of live trees (Luo et al., 2010; Zhu et al., 2011). The wood chips were screened to retain chips between 6 and 38 mm. The screen chips were frozen at approximately −16 °C until use.

A commercial cellulase cocktail, Cellic CTec 2, was generously provided by Novozymes North America (Franklinton, NC). Sodium acetate, sulfuric acid, and sodium bisulfite were all ACS reagent grade from Sigma–Aldrich (St. Louis, MO). All other chemicals, including culture media ingredients, were purchased from Fisher Scientific (Hanover Park, IL).

2.2. Microorganism and culture

Saccharomyces cerevisiae YRH400 is an engineered fungal strain for xylose fermentation with the needed genes integrated into the genome (Hector et al., 2011). The strain was grown at 30 °C for 2 days on YPD agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar. A colony from the plate was transferred by loop to liquid YPD medium in a flask and cultured for 2 days at 30 °C with agitation at 90 rpm on a shaking bed incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA). The cultured medium was used to inoculate the fermentation culture.

2.3. Substrate production by SPORL

SPORL pretreatment of wood chips was conducted using a 23-L laboratory wood pulping digester as described previously (Zhu et al., 2009). The digester was externally heated using a steam jacket and rotated at 2 rpm for mixing. Pretreatment was conducted at 180 °C using dilute sulfuric acid and sodium bisulfite solutions and 2 kg oven-dry (od) wood chips with a liquor to wood ratio (L/W) of 3:1. The sulfuric acid and bisulfite charge on od wood was 2.2 and 8 (wt/wt)%, respectively. The digester temperature was raised to 180 °C in approximately 15 min and maintained for another 20 min. After digestion, the pretreated wood chips remained intact and were disk-milled with the pretreatment spent liquor along with some fresh water (2.26 L/kg untreated wood) to facilitate milling and material transport after milling (Fig. 1). The disk plates had a pattern of D2B-505 and the disk plate gap was set at 1.0 mm. The solid and liquor fractions were separated after disk milling by pressing in a screened box. The moisture of the solid fraction was determined gravimetrically by oven drying the collected wet sample.

2.4. Pretreatment spent liquor concentration and conditioning

To conduct high solids enzymatic saccharification using the combined pretreated solids and spent liquor with up to 20% solids, the spent liquor, collected after disk milling was concentrated using a vacuum rotary evaporator (Rotavapor-R type WB, Buchi, Switzerland). The evaporation temperature was set to approximately 52 °C to avoid sugar degradation. The volumetric and mass concentration ratios of 6.25 and 5.42, respectively, were determined based on the initial and final liquor volumes and weights (Table 1). The pH of the concentrated liquor was adjusted to10 using solid calcium hydroxide.

2.5. Fed-batch saccharification solids and combined fermentation with pretreatment liquor

Different amounts of concentrated and conditioned liquor were used in seven fed-batch quasi-simultaneous saccharification and combined fermentation (qSSCombF) experiments as listed in Table 2. The liquor was first added to a fixed amount of pretreated solid substrate, and the mixture was adjusted to pH to 6.2 with solid calcium hydroxide. Acetic acid/sodium acetate buffer (50 mM) was added into the pH-adjusted mixture to conduct enzymatic hydrolysis at pH 5.5 using CTec2 at 12 FPU/g od unwashed solid substrate, or 9 FPU or 0.06 mL/g (0.07 g/g) of untreated wood. This elevated pH 5.5, higher than pH 5.0 recommended by Novozymes and exclusively used in the literature, is based on our previous studies that showed significant enhancement of enzymatic saccharification of SPORL-pretreated lignocellulosic substrates (Lan et al., 2012; Wang et al., 2012; Zhang et al., 2012). Hydrolysis was conducted in 125-mL flasks on a shaker/incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 50 °C and 200 rpm for 4 h to partially liquefy the solid substrate and allow for convenient sampling. The hydrolysate mixture was cooled to room temperature, inoculated with 2 mL of yeast seed, and incubated at 35 °C and 100 rpm. The pretreated solids were batch fed periodically according to the schedule listed in Table 2. The amounts fed and feeding times varied so that new feeding was made only after the existing solids were liquefied. The fermentation was terminated after 168 h. The fermentation broth was sampled periodically. Samples were always taken just before feedings and only when pretreated solids were liquefied as determined by visual observation. The fermentation broth samples were centrifuged at 12,000*g* for 5 min. The supernatants were stored at −16 °C for later analyses of ethanol, sugars, and inhibitors.

Duplicate saccharification and fermentation experiments were conducted for selected Runs (No. 1, 2 and 5 listed in Table 2) to verify the experimental repeatability.

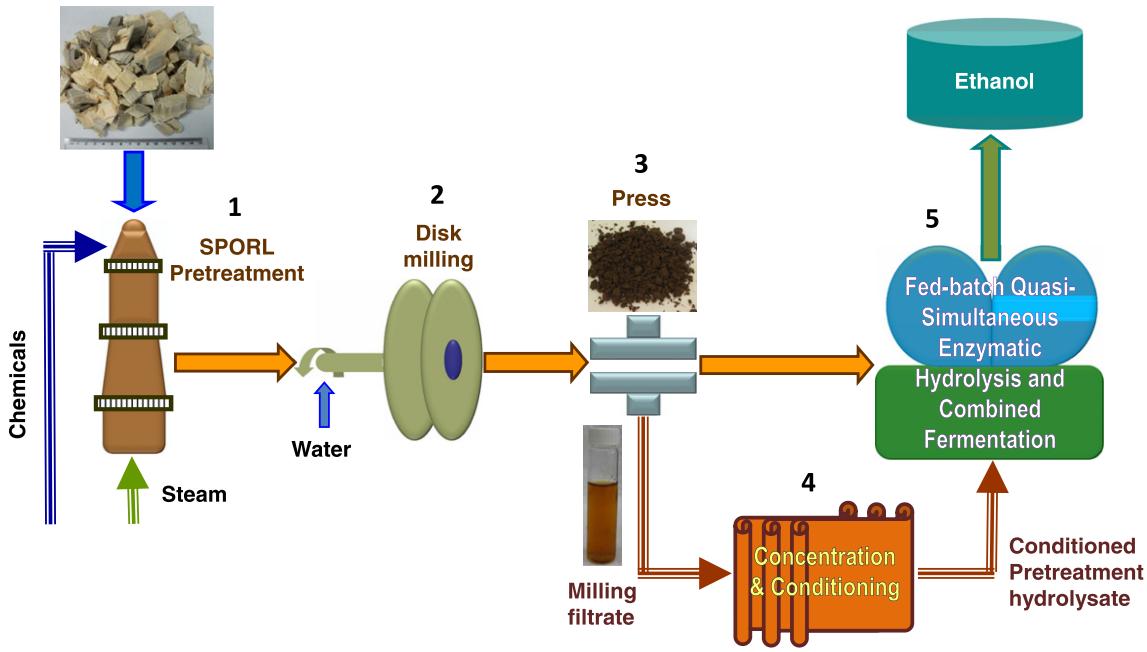

Fig. 1. Schematic flow diagram of experiments from SPORL pretreatment of wood chips, disk-milling, to fed-batch fermentation.

Table 1
Effects of concentrating and conditioning SPORL-pretreatment spent liquor on resultant spent liquor composition.

	Unconcentrated liquor	Concentrated liquor (Measured)	Concentrated liquor (Calculated)	Difference (%)
Volume (mL)	1000	160	NA	
Weight (g)	1000	184.5	NA	
Density (g/mL)	1.03	1.12	1.188	−5.72
Total solids (wt%)	6.5			
Arabinose (g/L)	0.92 ± 0.02	5.62 ± 0.37	5.76	−2.43
Galactose (g/L)	2.27 ± 0.05	13.03 ± 0.31	14.18	−8.11
Glucose (g/L)	5.85 ± 0.34	36.20 ± 1.58	36.56	−0.98
Xylose (g/L)	2.99 ± 0.12	17.95 ± 0.63	18.71	−4.06
Mannose (g/L)	8.01 ± 0.20	47.04 ± 1.33	50.06	−6.03
Furfural (g/L)	0.26 ± 0.01	0.51 ± 0.02	1.63	−68.71
HMF (g/L)	1.24 ± 0.08	6.38 ± 0.49	7.75	−17.68
Acetic acid (g/L)	2.67 ± 0.13	6.62 ± 0.19	16.69	−60.34

2.6. Analytical methods

The chemical compositions of the untreated wood and the SPORL pretreated substrate were measured by the Analytical and Microscopy Laboratory of the Forest Products Laboratory as described previously (Luo et al., 2010).

Sugar (glucose, arabinose, galactose, xylose and mannose) concentrations in the pretreatment spent liquor were determined using a Dionex HPLC system (ICS-3000) equipped with an integrated amperometric detector and Carbopac™ PA1 guard and analytical columns at 20 °C. Eluent flowrate was 0.6 mL/min, according to the following gradient: 0–25 min, 100% water; 25–35 min, 30% water and 70% 0.1 M NaOH; 35–40 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 post-column eluent.

Inhibitor (acetic acid, furfural, and HMF) concentrations in the pretreatment spent liquor were analyzed using the same Dionex HPLC system (ICS-3000) equipped with a Supelcogel C-610H column at 30 °C and a UV detector at 210 nm. Eluent was 0.1% phosphoric acid at a rate of 0.6 mL/min.

To determine monomeric sugars and inhibitors in the unwashed pretreated acidic solid fraction, the solid substrate was diluted to 4% total solids consistency and well mixed. The diluted solution was centrifuged at 12,000*g* and the supernatant was analyzed using the procedures described above. The data were then converted to wt% of unwashed solid or untreated wood base.

Ethanol was measured using an HPLC system (model L-2490, Hitachi High-Technologies Corporation, Japan) equipped with a BioRad (Hercules, CA) Aminex HPX-87H column (300 × 7.8 mm) operated at 55 °C. Dilute sulfuric acid solution of 5 mM was used as eluent at a flow rate of 0.3 mL/min. Sample injection volume was 20 μL. Refractive index detection was used to quantify ethanol through calibration. Samples were diluted in deionized water, and filtered through PrepSEP C18 (Fisher Scientific) filters prior to injection.

3. Results and discussion

3.1. Wood component recovery through SPORL pretreatment

The wet weight and moisture content of the initial wood chips, SPORL pretreated unwashed acidic solid fraction, and pretreatment spent liquor are listed in Table 3. The total solids recovery was 94.5% with 74.2% in the unwashed solids fraction and 20% in the spent liquor as soluble and suspended solids. Overall recoveries

Table 2
Schedules for different fermentation Runs using batch-fed wet solids of moisture 23.6%.

Run No.	Total dry solids (g); wet weight (g) of concentrated liquor	Amount of unwashed solid substrate fed at each time point: dry weight (g)					Initial total solids (%)	Calculated final total solids (%)
	0 h	0 h	24 h	48 h	72 h	96 h	0 h	96 h
1	0.84; 2.38	2.5	1.42	1.42	0	0	13.4	16.7
2	1.46; 4.15	2.5	1.42	1.42	0	1.42	15.9	19.2
3	1.31; 3.71	2.5	1.77	0	1.77	0	15.2	18.5
4	1.46; 4.15	2.5	2.12	0	0	2.12	15.9	19.3
5	1.26; 3.56	3.0	1.42	0	1.42	0	16.4	18.8
6	1.42; 4.02	3.0	1.77	0	0	1.77	17.0	19.5
7	1.57; 4.45	3.0	2.12	0	0	2.12	17.6	20.1

Table 3
Mass, solids contents, and chemical compositions (in wt% of untreated wood) of the untreated wood, pretreated solids, and pretreated spent liquor.

	Untreated wood	Unwashed solid substrate[a]	Unconcentrated liquid (spent liquor)[a]	Total recovery (%)
Total mass (kg)	2.27	6.29	6.23	
Moisture (%)	11.7	76.4	93.5	
Total solids (kg)[b]	2.00	1.48; 74.2%	0.40; 20%	94.5
Klason lignin (%)	28.6	23.4; 81.7%	5.2; 18.3% (by balance)	100.0
Arabinan (%)	1.7	0.6 ± 0.00; 34.2%	0.3; 17.8%	52.0
Galactan (%)	2.9	1.6 ± 0.04; 54.0%	0.7; 25.5%	79.5
Glucan (%)	41.9	36.6 ± 1.01; 87.4%	1.5; 3.7%	91.1
Xylan (%)	5.5	2.9 ± 0.09; 52.5%	0.9; 16.9%	69.4
Mannan (%)	11.7	6.6 ± 0.22; 56.6%	2.4; 20.7%	77.3
Furfural (%)[c]		0.1 ± 0.01; 2.0%	0.1; 2.0%	4.1
HMF (%)[c]		0.5 ± 0.00; 4.4%	0.5; 4.2%	8.6
Acetic acid (%)		3.25 ± 0.11	0.8	

[a] The numbers after ";" is wt% of theoretical based upon untreated wood carbohydrate content.
[b] In oven dry (od) weight.
[c] Reported as of pentosan and hexosan for furfural and HMF, represent percent of pentosan and hexosan converted to furfural and HMF, respectively.

of glucan, xylan, and mannan were 91.1%, 69.4%, and 77.3%, respectively. Most of the glucan (87.4%) was retained in the unwashed solids. Over two thirds of recovered xylan and mannan was present in the unwashed solids. Based on the analysis of a sample of washed pretreated solids, most of the recovered xylan and mannan were in the form of monomeric sugars, i.e., xylose and mannose, and less than 10% of them remained as polysaccharides. Approximately 50% of the furfural and HMF produced during pretreatment was found in the unwashed solids, while over 75% of the acetic acid remained with the solids, suggesting that the unwashed solids contained more than the half of the dominant fermentation inhibitors.

3.2. Effect of liquor conditioning on sugar and inhibitor profile

The major purpose of liquor concentration through vacuum evaporation was not to reduce inhibitors, but rather to remove water for high titer ethanol production when combining the pretreatment spent liquor and enzymatic hydrolysate. Nevertheless, evaporation reduced the amount of inhibitors as revealed by the difference between the measured and calculated inhibitor concentrations in the concentrated liquor (Table 1). The calculated inhibitor concentrations were determined from the volumetric concentration factor of 6.25 (=1000/160) and the inhibitor concentrations in the unconcentrated liquor (Table 1). The reduction in HMF was minimal at approximately 15% (or HMF recovery was 85%); however, the reductions in furfural and acetic acid were over 60% (or recovery less than 40%) due to vaporization. Because approximately 50% of the furfural and HMF and 75% of the acetic acid were found in the unwashed solids (Table 3), a net reduction of HMF, furfural and acetic acid of 8, 30, and 15%, respectively occurred when solids and concentrated liquor were recombined. The reduction of 30% in furfural is not critical because furfural is a minor inhibitor from SPORL pretreatment of lodgepole pine (softwood) with concentration in the concentrated liquor of only 1.6 g/L (Table 1). Therefore, evaporation did not significantly reduce inhibitors.

The low temperature evaporation did not degrade sugars in the pretreatment spent liquor. The differences between the measured and calculated sugar concentrations in the concentrated liquor were each within one standard deviation except for mannose with a 6% difference with relative standard deviation of 3%.

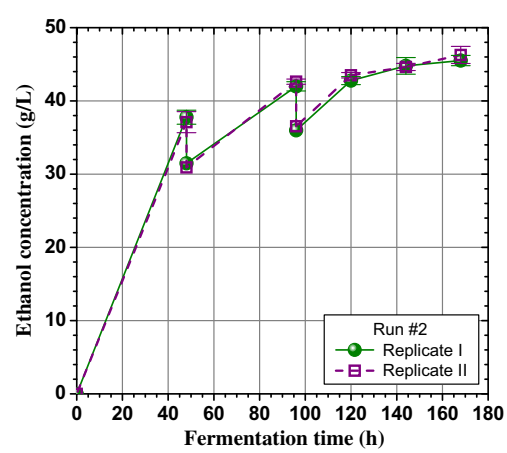

Fig. 2. Repeatability of time-dependent ethanol concentration from two duplicate experiments of enzymatic saccharification and combined fermentation for Run #2. The means (data) and standard deviations (as error bars) were from two HPLC determinations for each fermentation test.

Table 4
Ethanol concentration and wet weight of the fermentation broth, and ethanol productivity and yield from fermentation runs.

Run No.	Ethanol (g/L)[a]; total fermentation broth wet weight (g)					Ethanol yield @ 48 h (g/g sugar)[b]	Maximal ethanol yield (g/g sugar)[b]	Maximal ethanol yield (L/tonne wood)	Ethanol titer at maximal yield (g/L)
	48 h	72 h	96 h	120 h	144 h				
1	36.1 ± 0.8 31	41.1 ± 1.0 37	40.0 ± 1.5 37			0.38 (75.1)	0.40 (78.4)	287	41.1
2	37.4 ± 1.2 31		42.3 ± 0.5 37	43.1 ± 0.5 42	44.7 ± 0.8 42	0.38 (73.8)	0.39 (76.6)	280	45.9
3	34.9 ± 0.6 32.5	39.4 ± 0.6 32.5			42.8 ± 1.4 40	0.35 (67.6)	0.38 (74.4)	272	42.8
4	34.1 ± 1.2 34	39.9 ± 0.3 34	46.3 ± 0.8 34		39.7 ± 1.3 43	0.33 (63.6)	0.34 (66.4)	243	39.7
5	35.4 ± 1.2 32	40.6 ± 1.2 32		43.2 ± 1.0 38	44.9 ± 0.8 38	0.34 (65.8)	0.39 (76.7)	281	44.9
6	38.1 ± 1.5 33.5		46.0 ± 0.9 33.5		47.4 ± 0.6 41	0.35 (68.3)	0.40 (77.9)	285	47.4
7	38.6 ± 1.3 35		46.7 ± 0.9 35		45.1 ± 0.8 44	0.34 (67.1)	0.37 (71.8)	263	45.1

[a] Sampled withdrawn before feeding further solids. Standard deviations were calculated from two HPLC determinations (four measurements for Runs #1, #2, and #5 with replicate fermentation tests).
[b] Based on the total of glucan, xylan, and mannan in the solids and glucose, xylose, and mannose in the pretreatment spent liquor. The numbers in the parenthesis are percent of theoretical (0.511 g/g sugar).

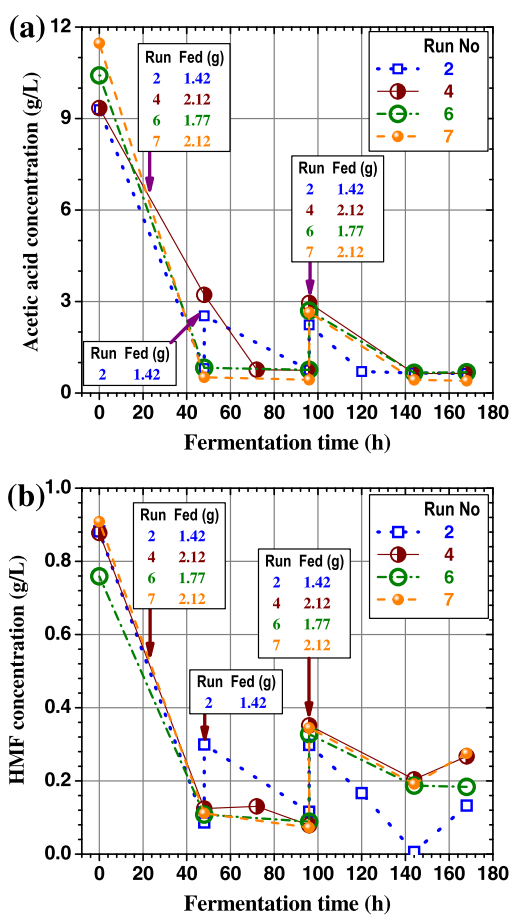

Fig. 3. Time-dependent inhibitor concentration measured in the fermentation broths from four selected runs. (a) Acetic acid; (b) HMF.

3.3. Quasi-simultaneous enzymatic saccharification and combined fermentation (qSSCombF)

The replicate fed-batch qSSCombF experiments were conducted for Runs 1, 2, and 5 (Table 2). The time-dependent ethanol concentration data show excellent repeatability as shown in Fig. 2 for Run #2. The differences in ethanol concentration were well within the ethanol measurement standard deviations as shown in Table 4 based on four measurements, i.e., duplicate HPLC determinations of each of the replicate fermentation test. Similar repeatability was also observed from Run # 1 and #5.

The time-dependent ethanol concentrations for the seven runs are listed in Table 4 along with wet weights of the samples after batch feedings. Ethanol titers of greater than 40 g/L were achieved for most of the runs after 96 h of fermentation. Most of the runs achieved ethanol yields over 270 L/tonne of wood. Run #1 achieved the highest ethanol yield of 287 L/tonne of wood with a saccharification and fermentation yield of 78.4% of theoretical and an ethanol concentration of 41.1 g/L. Run #1 had the lowest final total solids loading of 16.7% (Table 2) which facilitated insoluble solids liquefaction and saccharification. When comparing Runs #2 and #4, the final total solids were almost the same but the solids were fed into the reactor differently, i.e., Run #2 was fed with smaller doses of 1.42 g three times while Run #4 was fed larger doses of 2.12 g twice. Frequent feeding of a smaller amount (Run #2) produced better liquefaction which resulted in a higher ethanol yield of 280 L/tonne of wood and a saccharification and fermentation yield of 76.6% of theoretical. In comparison, 243 L/tonne of wood or 66.4% of theoretical were achieved in Run #4 where biomass was fed less often at higher doses. The difference in inhibitor concentrations between the two runs does not appear to explain this discrepancy (discussed below). The observed differences between Run #2 and #4 were caused by the difference in the rate of solids liquefaction and saccharification. To further illustrate this point, Run #6 with #7 can be compared. Both runs were fed twice; however, Run #6 was fed 1.77 g each time while Run #7 was fed 2.12 g each time. Although total solids loading for Run #6 of 19.5% was slightly lower than the 20.1% for Run #7, Run #6 produced a higher ethanol yield of 285 L/tonne of wood (77.9% of theoretical) at a higher titer of 47.4 g/L than Run #7 with 263 L/tonne of wood (71.8% of theoretical) at 45.1 g/L. Similar comparisons can be made between Runs #3 and #5 where Run #5 was fed 1.42 g each time while Run #3 was fed 1.77 g each time. The final total solids loadings were almost the same for the two runs. However, Run #5 produced a higher ethanol yield of 281 L/tonne of wood (76.7% of theoretical) at a higher titer of 44.9 g/L than the 272 L/tonne (74.4% of theoretical) at 42.8 g/L from Run #3.

Yeasts respond to the presence of furans by reducing them to less toxic compounds and to acetate by metabolizing it. Inhibitors were monitored during the fermentation to determine whether or not batch feeding led to accumulation of these inhibitors. The

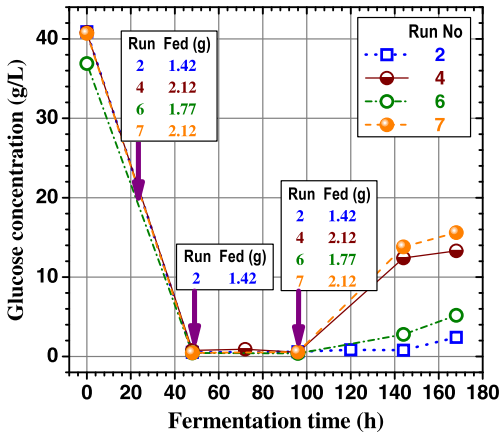

Fig. 4. Time-dependent glucose concentration measured in the fermentation broths from four selected runs.

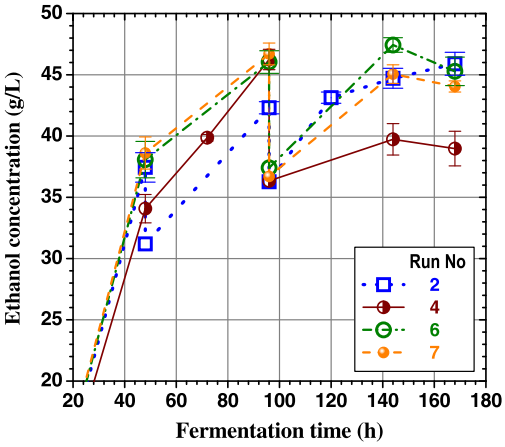

Fig. 5. Time-dependent ethanol concentration measured in the fermentation broths from four selected runs.

acetic acid concentration started at approximately 10 g/L and rapidly declined in concentration within the first 48 h of fermentation to approximately 1 g/L even when the fed-batch was dosed at 24 h (Fig. 3a). The differences among the fermentation runs were not significant. A similar trend was also observed for HMF (Fig. 3b), i.e., HMF concentration decreased from approximately 1 g/L to approximately 0.1 g/L after 48 h. The increases in acetic acid and HMF concentrations right after feeding were from the inhibitors in the fed solids. These results indicate that acetic acid and HMF concentrations were low. In all of the fed batch runs, yeast biotransformation of these two inhibitors was unconstrained by culture conditions. These two inhibitors should not affect yeast performance in terms of ethanol productivity. The furfural concentration was very low and below the detection limit throughout the fermentation.

To illustrate that the observed differences in the maximal ethanol productivities among the seven fed-batch runs were due to differences in solids liquefaction and enzymatic saccharification, the time-dependent glucose concentrations in the fermentation broth from four different runs were compared (Fig. 4). Run #2 with more frequent feeding in smaller doses had less residual glucose than Run #4 despite similar final solids. The higher terminal glucose concentration of Run #4 resulted in a lower ethanol production than that of Run #2 (Table 4) as discussed above. Similar conclusions regarding the advantages of more frequent feedings at smaller doses can be made by comparing Runs #6 and #7, as well as Runs #3 and #5 (not shown in Fig. 4).

The ethanol profiles mirror the glucose concentration data. Run #4 and #7, exhibited significantly increased glucose concentrations after 96 h (Fig. 4), also showed significant declines in ethanol concentrations after 96 h partially due to dilution by feeding wet solids (step decrease shown in Fig. 5). While for Run #2, which had a minimal increase in glucose concentration after 96 h (Fig. 4), the ethanol concentration recovered from the dilution-induced decrease rapidly and resulted in an increased terminal ethanol concentration after the feeding at 96 h (Fig. 5). Comparing Run #6 with Run #2, Run #6 had a slightly higher terminal glucose concentration and a slight decrease in terminal ethanol concentration after the feeding at 96 h. Runs #3 and #5 also had similar trends (not shown). The superiority in yields associated with a more frequent feeding schedule can be attributed to several causes,

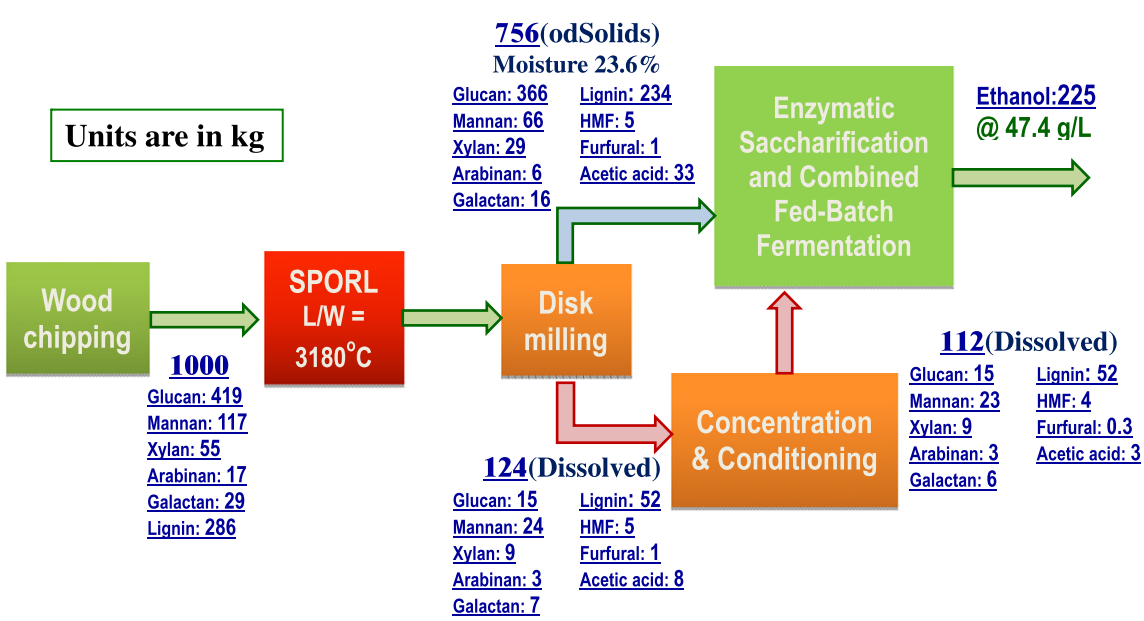

Fig. 6. Block diagram showing component mass balance. Unless indicated, units are in kilograms.

including a more even continuous release of glucose, more gradual increases in organic inhibitors and salts, which are related to osmotic stress, and maintenance of a better mixing regime. In particular, it has been observed that a gradual release of glucose favors xylose fermentation for *S. cerevisiae* strains engineered of xylose fermentation. It would be interesting to evaluate if this trend continues when implementing a continuous feeding scheme.

3.4. Mass balance

The mass balance of each component throughout the entire process for the best fermentation Run #6 is shown in Fig. 6. Most of the data were taken from Table 3. Furfural and HMF were expressed as pentosan and hexsan, respectively. All sugars were also expressed as pentosan or hexsan for easy mass balance calculation. All data were expressed on a 1000-kg basis. The results indicated an ethanol yield of 225 kg/tonne of wood or 285 L/tonne of wood.

4. Conclusions

This study demonstrates an integrated process for ethanol production at high titers and yields from lodgepole pine, using SPORL pretreatment in combination with quasi-simultaneous enzymatic saccharification and combined fermentation (qSSCombF). A commercially viable ethanol titer of 47.4 g/L was achieved with a yield of 285 L/tonne wood when qSSCombF was conducted at a total solids loading of 19.5% through a fed-batch process using *S. cerevisiae* YRH400 and a commercial cellulase CTec2 loading of 9 FPU/g (or 0.06 mL/g) of untreated wood. Inhibitors (acetic acid and HMF) did not affect ethanol production. Ethanol production is limited by the rate of solids liquefaction and enzymatic saccharification. Future study will target eliminating liquor evaporation and focus on optimization of continuous fed-batch for high efficiency ethanol production.

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