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Optimization of Combined Clean Fractionation and Hydrothermal Treatment of Prairie Cord Grass

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ABSTRACT: A clean sequential process including clean fractionation and hydrothermal post-treatment was developed to fractionate and pretreat prairie cord grass (PCG) prior to ethanol fermentation. An optimized clean fractionation was applied to extract lignin; then, the remaining material was subjected to a hydrothermal treatment process to enhance cellulose digestibility. The main purpose of the hydrothermal post-treatment was to improve digestibility of delignified cellulose and reduce harshness of the clean fractionation process. Optimization was performed for solvent compositions as well as time and temperatures applied to each sequential process. The glucose yield was 89% from enzymatic hydrolysis and 92% from combined fractionation and hydrothermal treatments of PCG.

1. INTRODUCTION

The search for an effective and economically feasible lignocellulose pretreatment method is gaining more attention among researchers. It has been reported that there are several chemical treatments that have been applied to lignocellulosic biomass with good cellulose-to-glucose conversion results.[1−4] An alternative to the use of chemicals in the lignocellulosic biomass treatment is utilization of water at high temperatures without the addition of catalysts, which is known as hydrothermal pretreatment.[5,6] Water at high temperatures (~200 °C) is acidic, acting as a catalyst for biomass disruption[7] and eliminating the need for a catalyst. However, any coproducts produced from these treatments are of low economical value. Using high temperatures and harsh chemicals, a large portion of hemicellulose is decomposed to furfurals and the lignin fraction is subjected to structural degradation, making it only suitable for combustion.[8]

Organosolv treatment is a process that gives new opportunities in coproducts utilization, since the coproducts are mostly of high purity. Fractionation of biomass uses differences in the affinity of lignocellulose components toward different solvents. Organic solvents dissolve lignin, whereas carbohydrate components are dissolved in water or remained in the solid. Organosolv lignin is high in phenolic hydroxyl groups, which are essential for a variety of chemical products, because of their high antioxidant activity.

High temperatures (over 160 °C) in the organosolv treatment can be reduced by catalyst addition (e.g., sulfuric acid), which triggers the lignocellulose breakdown by reducing pH.[9] However, using a catalyst is always associated with the need for its recovery and neutralization of aqueous fractions. In the U.S. National Renewable Energy Laboratory (NREL)'s procedure of clean fractionation, methyl isobutyl ketone (MIBK) was the lignin solvent of choice. An organic-aqueous solvent (MIBK and water) was used to remove lignin and hemicellulose from the biomass, leaving cellulose in the solid, and therefore providing three relatively "clean" fractions.[9] There are many solvents that can be used in the clean fractionation, including ethanol, methanol, organic acids, and esters.[10] Esters, especially ethyl acetate, are relatively simple to produce, nontoxic, and inexpensive, and they can represent an interesting alternative solvent to toxic and expensive ketones.

Organosolv treatment may be combined with other treatments to reduce the harshness of the process (e.g., decreasing reaction temperature and eliminating catalysts) and enhance cellulose digestibility[10] as well as lignin recovery.[11] According to Rughani et al.,[11] good results can be achieved with applications of other treatments before organosolv fractionation. However, application of any type of hydrothermal pretreatment before the use of organic solvents can decrease delignification yields. This is due to hydrophobic lignin forming residual precipitation on the cellulose fibers. Hydrophobic lignin becomes irreversibly bonded to the cellulose and also affects cellulose enzymatic digestibility.[12] In order to enhance cellulose digestibility after organosolv delignification, it seems to be more effective to apply a post-treatment (e.g., hydrothermal) that has been proven to produce high conversion yields for raw lignocellulosic materials.[5,6] Cellulose digestibility in herbaceous biomass was reported to be higher after the hydrothermal treatment, compared to organosolv treatment, which suggests the applicability of a post-treatment after the organosolv process.[13]

Applying a hydrothermal post-treatment instead of simply increasing the temperature of clean fractionation is more advantageous, because of the desire to preserve extracted lignin in its least-altered form. A low-temperature delignification ensures that the lignin structure can be thermally unaltered and limits the cross contamination of fractions by dissolved carbohydrates and released byproduct, which happens at higher temperatures. The objectives of this study were to optimize hydrothermal processing conditions on clean fractionated

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Table 1. Results of Clean Fractionation Optimization

	Conditions						
expt	temperature [°C]	time [min]	ethyl acetate content [% w/w]	ethanol content [% w/w]	lignin recovery [%]	hydrolysis glucose yield [%]	xylose aqueous fraction yield [%]
1	110	10	15	10	2.86 ± 0.00	35.50 ± 0.17	20.00 ± 0.88
2	140	10	15	10	5.70 ± 1.34	42.19 ± 2.25	25.56 ± 2.53
3	110	30	15	35	10.46 ± 2.06	35.18 ± 3.99	19.15 ± 0.89
4	140	30	15	35	13.53 ± 0.95	41.61 ± 1.74	20.73 ± 0.90
5	110	30	50	10	13.09 ± 1.67	36.52 ± 1.07	16.37 ± 0.52
6	140	30	50	10	16.41 ± 1.65	41.11 ± 0.52	18.75 ± 0.31
7	110	10	50	35	23.02 ± 0.31	34.04 ± 1.46	16.72 ± 0.07
8	140	10	50	35	19.23 ± 1.67	36.25 ± 2.00	19.29 ± 0.38
9	125	20	33	23	17.60 ± 2.02	39.63 ± 1.76	19.86 ± 0.43
10	125	20	33	23	16.84 ± 1.64	39.50 ± 1.76	18.65 ± 0.45
11	125	20	33	23	13.54 ± 4.42	40.47 ± 3.73	17.39 ± 2.54
12	125	20	33	23	17.83 ± 0.29	39.85 ± 1.08	19.52 ± 0.11
13	125	20	62	23	15.94 ± 3.01	37.86 ± 4.30	16.04 ± 1.05
14	125	20	3	23	6.19 ± 0.68	40.03 ± 3.35	21.79 ± 0.08
15	125	20	33	44	18.56 ± 4.05	36.71 ± 1.56	21.77 ± 1.60
16	125	20	33	2	14.00 ± 1.74	36.64 ± 1.59	25.58 ± 2.00
17	150	20	33	23	15.21 ± 6.03	41.90 ± 0.14	22.32 ± 2.26
18	100	20	33	23	14.25 ± 1.33	35.64 ± 2.37	18.17 ± 0.44
19	125	37	33	23	19.73 ± 0.97	38.24 ± 2.34	21.15 ± 0.04
20	125	3	33	23	18.03 ± 2.70	40.56 ± 0.90	17.60 ± 0.49

prairie cord grass (PCG) and evaluate the sequential process effect on the digestibility of cellulose-rich fractions.

2. MATERIALS AND METHODS

2.1. Compositions of Prairie Cord Grass. Prairie cord grass (PCG) was harvested in Brookings, SD. Compositional analysis of the PCG was performed via acid hydrolysis, according to Sluiter et al.[14] The results (per dry weight) were as follows: 36.70% ± 0.01% glucose, 13.52% ± 2.00% xylose, 1.59% ± 0.57% arabinose, 1.40% ± 0.5% galactose, 0.30% ± 0.00% mannose, 20.96% ± 0.52% lignin, 5.65% ± 0.04% ash, and 19.88% ± 0.01% extractives.

2.2. Clean Fractionation. Prior to clean fractionation, PCG was ground to pass through a 1-mm screen (Thomas–Wiley Laboratory Mill, Model 3375-E15, Thomas Scientific, USA). Clean fractionation was performed in pressure reactors (custom-made stainless steel tubes with screw caps and volume capacity of 250 mL) with 10 g dry matter (DM) of biomass loading in 100 g of the solvent–biomass mixture. The process was conducted by controlling temperatures using LabView version 8.2 and monitoring pressures with top-mounted pressure gauges.

The solvent was composed of ethyl acetate, ethanol, and deionized water in different ratios (see Table 1). Ethyl acetate was chosen to replace MIBK in standard clean fractionation,[9] because of its low toxicity (NFPA Health Hazard is 1, while that for MIBK is 2). The reaction temperatures ranged from 110 °C to 140 °C with processing times of 10–37 min.[15] The reactors were placed in an insulated heating block and heated until reaching the desired temperature (heating time was 20 min). After reaction, the reactors were cooled in a cold water bath for ∼20 min.

Clean fractionation was performed in order to extract lignin and hemicellulose from the biomass and yield a cellulose-rich solid. The cellulose-rich solids fraction obtained from this step resulted in low glucose yields in the enzymatic hydrolysis, which suggested a post-treatment application.[15]

2.3. Hydrothermal Post-Treatment (HP) of Cellulose-Rich Solid Fraction. The cellulose-rich solids fraction obtained from clean fractionation was hydrothermally treated subsequently in the same custom-made reactor setup used for clean fractionation. The cellulose-rich solids fraction and deionized (DI) water were loaded into the reactors to produce 10% w/w of dry matter (DM) in 50 g of the biomass–water mixture. Two factors were optimized in the process: temperatures, in the range of 162–218 °C, and time, in the range of 8–22 min. The reactors were preheated for ∼40 min and cooled in a cold water bath after the process (∼1 h). After the hydrothermal treatment, the mixture was separated into liquid and solid fractions by vacuum filtration. Solid retentate that remained on the filter was collected and kept frozen for subsequent analysis and enzymatic hydrolysis. Filtrates obtained after vacuum filtration were analyzed for dissolved sugars (glucose, xylose) and byproduct (acetic acid, lactic acid, furfural, hydroxymethyl furfural (HMF)), according to LAP 013[16] and LAP 015,[17] using high-performance liquid chromatography (HPLC) system (Agilent Technologies, System 1200 with a Bio-Rad Aminex 87H Column) that was equipped with a refractive index detector (RID) device. The sample volume was 20 μL. A 0.005 M H_2SO_4 mobile phase was used at a flow rate of 0.6 mL/min at 65 °C.

2.4. Enzymatic Hydrolysis. Hydrolysis of the cellulose-rich solid fraction obtained after the integrated pretreatment process was performed according to NREL protocol.[18] The hydrolysis was conducted in a 100-mL mixture containing 3% w/w dry matter content and monitored by collecting 1.5 mL sample after 0, 3, 6, 12, 24, 34, 48, and 72 h. Hydrolysis was performed in duplicate using cellulase (Novozymes, NS50013) and β-glucosidase (Novozymes, NS50010) in amounts of 15 FPU/gDM (FPU = filter paper unit) and 60 CBU/gDM (CBU = cellobiase unit), respectively. Concentrations of sugars (glucose, xylose) and byproduct (acetic acid, lactic acid, furfural, HMF) in the enzymatic hydrolysates were measured using HPLC (Agilent Technologies System 1200 with Bio-Rad Aminex 87H Column) that was equipped with an RID device. A 0.005 M H_2SO_4 mobile phase was used at a flow rate of 0.6 mL/min at 65 °C. The sample volume was 20 μL. Samples were prepared according to LAP 013[16] and LAP 015.[17]

2.5. Glucose Yields. Glucose yields obtained during enzymatic hydrolysis were calculated according to the following equation:

$$\text{hydrolysis yield (\%)} = \frac{\text{glucose amount after hydrolysis}}{\text{glucose amount in raw material}} \times 100 \quad (1)$$

To assess the possibility of using the entire slurry after integrated treatments (without solid–liquid separation), the entire process efficiency

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was calculated using eq 2. This equation includes glucose dissolved in the filtrate obtained in the hydrothermal post-treatment.

$$\text{entire process efficiency (\%)} = \frac{(\text{glucose in solid}) + (\text{glucose in filtrate after HP})}{\text{glucose amount in raw material}} \times 100 \quad (2)$$

Xylose recoveries were calculated in the same manner:

$$\text{xylose recovery (\%)} = \frac{\text{xylose after hydrolysis}}{\text{xylose amount in raw material}} \times 100 \quad (3)$$

$$\text{filtrate xylose recovery (\%)} = \frac{\text{xylose in filtrate}}{\text{xylose amount in raw material}} \times 100 \quad (4)$$

2.6. Response Surface Analysis. The hydrothermal treatment experimental design was based on a central composite experimental design (CCD). A 2^2-factorial central composite design with four replications at the center point was used (Table 2), giving 12 experiments overall.

Table 2. Experimental Plan for Hydrothermal Treatment

			Solids Fraction Hydrolysis Glucose Yield		Total Pretreatment Efficiency	
expt	temperature [°C]	time [min]	value [%]	std dev	value [%]	std dev
1	162	15	50.02	3.57	60.52	0.88
2	170	10	51.13	3.11	60.90	0.83
3	170	20	55.44	1.07	63.43	1.99
4	190	8	84.10	3.28	88.52	4.65
5	190	15	79.74	3.38	83.66	3.37
6	190	15	81.76	1.77	85.76	0.57
7	190	15	83.37	1.31	87.17	0.52
8	190	15	82.06	2.45	86.43	0.66
9	190	22	84.94	1.28	88.53	0.08
10	210	10	88.63	1.48	92.08	2.28
11	210	20	85.51	1.01	88.80	0.32
12	218	15	80.23	1.42	83.93	1.94
BLIND			32.68	0.22		

2.7. Energy Recovery Efficiency. An estimate of the energy recovery efficiency (η) of the integrated process was calculated to evaluate its feasibility. All output streams were analyzed for energy content and compared to the raw material heating value (eq 5).

$$\eta = \frac{\text{heating value of output products}}{\text{heating value of input material}} \quad (5)$$

The output products included lignin extracted during clean fractionation and theoretical ethanol obtained from the fermentation of glucose and xylose. Ethanol yield was calculated theoretically (since fermentation was not performed in this study), using stoichiometric equations of glucose and xylose fermentation (see reactions 6 and 7).

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (6)$$

$$C_5H_{10}O_5 \rightarrow \frac{5}{3}C_2H_5OH + \frac{5}{3}CO_2 \quad (7)$$

3. RESULTS AND DISCUSSION

3.1. Clean Fractionation. Optimization of clean fractionation was performed with results presented in Table 1. Optimization was focused on maximizing lignin extraction and maintaining the lowest possible harshness of the processing conditions. The optimal conditions of clean fractionation resulted in 20% lignin recovery (based on the input material lignin content), 38% hydrolysis glucose yield, and 21% xylose recovery (experiment 19). Biomass delignification can be performed with the use of organic solvents at a lower temperature without the addition of a catalyst. However, lignin recovery obtained under optimal conditions (eliminating the catalyst and applying low temperature) was considered low, when compared to recent studies. Efficient organosolv treatments achieve lignin removal rates of 40%−70%.[19,20] Lignin yields from clean fractionation span between 18% and 32%, depending on the type of material.[21] Using green liquor extraction of hardwood chips, Luo et al. obtained ∼2.36%−2.94% of lignin from raw wood materials.[22] Garcia et al. implemented a similar process idea, but applied autohydrolysis (hydrothermal treatment) as the first step and used a high-temperature (175−200 °C) ethanol−water extraction as the second step. They achieved up to 67% lignin recovery in the organic fraction.[20] However, autohydrolysis causes lignin depolymerization and subsequent repolymerization with condensed hemicellulose residuals ("pseudo-lignin"), causing false Klason lignin measurements.[23,24] To avoid this phenomenon, this study implemented deligninfication as the first step of the integrated process. However, in this case, a large part of the hemicellulose and a part of the lignin remained bonded with the cellulose, which presumably still had a high crystallinity, a high degree of polymerization (DP), and most importantly, a low pore volume. All of these factors affect digestibility during hydrolysis. A maximum hydrolysis conversion of cellulose into glucose (yield of 42%) and the highest xylose recovery (26% in aqueous fraction) were found in experiment 2 with the temperature of 140 °C, a low solvent concentration, and a reaction time of 10 min (see Table 1). This indicates that hydrothermal post-treatment after organosolv delignification must be applied to improve cellulose digestibility.

3.2. Hydrothermal Post-Treatment. Hydrothermal post-treatment was applied to the cellulose-rich solids fraction obtained from the clean fractionation process using the predetermined optimal condition (125 °C, 37 min, with the ratio of ethyl acetate:ethanol:water = 32.5:22.5:45). Different temperatures and times of hydrothermal treatment resulted in different glucose yields, xylose recoveries, and byproduct generation. Glucose yields obtained from 12 different hydrothermal treatment experiments can be found in Table 2. The highest glucose yield was achieved from experiment 10 (89% from hydrolysis and 92% in the combined fractions from the entire treatment), which was carried out at 210 °C for 10 min. This result is comparable to other studies using hydrothermal treatment alone.[5,25,26]

Experiments with temperatures at or below 170 °C or above 210 °C resulted in lower glucose yields (experiments 1, 2, 3, and 12). Processing times longer than 10 min at 210 °C (experiment 11) resulted in a decrease in the hydrolysis yield (by ∼3%). A similar trend was observed in previous trials using hydrothermal treatment applied to PCG alone.[5] Experiments 5−8 (center points) produced hydrolysis yields of 80% and more. Final concentrations of glucose after hydrolysis termination can be found in Table 3. Monitored glucose production during hydrolysis is shown in Figure 1 for experiments 8 and 10, which show that hydrolysis could be terminated at 48 h and maximum glucose yields still could be obtained.

Xylose released during enzymatic hydrolysis (3−58%) was generally high compared to the xylose obtained from clean fractionated samples without hydrothermal post-treatment

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Table 3. Glucose Final Concentrations

	Glucose Concentration after Hydrolysis [g/L]		Glucose Concentration in Filtrate [g/L]	
expt	value	std dev	value	std dev
1	8.41	0.97	0.92	0.37
2	8.84	0.74	0.90	0.40
3	10.69	1.18	1.03	0.54
4	17.15	1.35	1.05	0.18
5	17.29	2.98	1.28	0.49
6	17.41	1.29	1.15	0.08
7	17.82	1.52	1.35	0.07
8	16.93	0.28	1.14	0.28
9	18.50	1.24	1.84	0.00
10	19.03	1.98	3.27	0.61
11	18.81	2.09	2.53	0.63
12	18.09	2.69	1.82	0.84

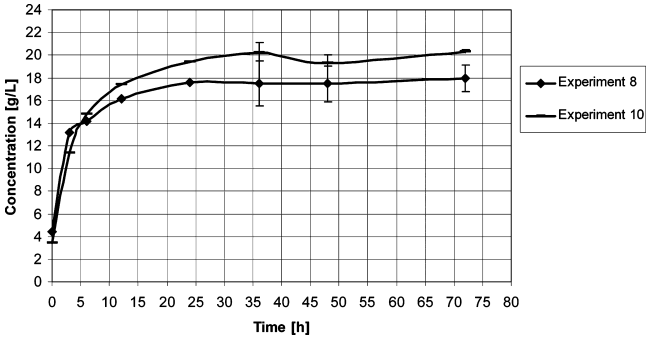

Figure 1. Glucose production during enzymatic hydrolysis.

(26%). However xylose recovery in the posthydrothermal filtrate fraction was lower by at least half when compared to the extracted xylose recovery in clean fractionation which reached 21%.

Xylose recovery results can be found in Table 4. The plot of xylose production during the at-line monitored hydrolysis for experiments 8 and 10 can be found in Figure 2. The highest xylose recovery during hydrolysis was achieved under the conditions of experiments 1 through 3 where the temperature was the lowest (162−170 °C). Xylose yields tended to decrease with increasing temperatures. Therefore, to achieve simultaneous high xylose and glucose yields from hydrolysis, the conditions in experiments 5−8 (the center point of the experimental design) would be preferable (190 °C and 15 min). These conditions achieved 37% of xylose recovery during enzymatic hydrolysis and produced an overall recovery of 58% (including xylose yields from the prior clean fractionated aqueous fraction).

Table 4. Xylose Recovery after Hydrothermal Treatment

	Xylose Recovery in Hydrolysis of Solids Fraction [%]		Xylose Recovery in Filtrate [%]	
expt	value	std dev	value	std dev
1	57.50	0.00	1.71	0.78
2	51.94	7.90	2.04	0.94
3	50.79	2.19	3.18	0.64
4	39.61	3.53	9.73	0.24
5	46.97	0.00	4.99	0.00
6	35.17	5.98	13.52	3.17
7	33.27	6.27	10.21	0.00
8	37.12	1.91	11.72	1.08
9	28.67	2.24	13.11	0.00
10	13.53	3.21	11.75	7.58
11	8.51	2.42	5.01	3.35
12	2.57	3.64	3.89	3.38

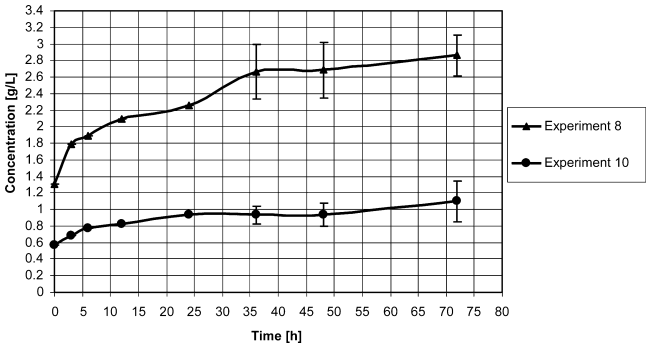

Figure 2. Xylose production during enzymatic hydrolysis.

The results of byproduct formation during enzymatic hydrolysis and its concentration in the hydrothermal filtrates can be found in Table 5. The generation of by-products during hydrothermal post-treatment was much higher than that during clean fractionation, because of the high processing temperatures that were used. The measured by-products included acetic acid, lactic acid, furfural, and hydroxymethyl furfural (HMF). The majority of byproduct was transferred into the hydrothermal filtrate, which was not rich in xylose or glucose and, therefore, could be discarded.

The concentration of byproduct in the hydrothermal filtrate (Table 5), as well as in the enzymatic hydrolyzate, was dependent on the process temperature during the hydrothermal post-treatment. This same trend did not apply to the acetic acid concentration after enzymatic hydrolysis, which was fairly invariant throughout the experiments. However, byproduct concentrations in the hydrolyzate were still low, which suggested that they would not inhibit yeast performance. Acetic acid above 0.05% w/v inhibits yeast growth. Concentrations of this byproduct ranged from 0.043% to 0.066% w/v in the 12 experiments. The chosen conditions of experiments 5−8 and 10 showed acetic acid concentrations of 0.050% and 0.046% w/v, which are acceptable. The maximum lactic acid concentration that can be tolerated by yeast is 0.8%; however, stress was observed for concentrations higher than 0.2%. The highest concentration of lactic acid that occurred during hydrolysis was 0.027% (experiment 7), which was well below the inhibitory value. In all experiments, the lactic acid concentration in the filtrate was acceptable; however, the acetic acid concentrations exceeded acceptable values.[27]

Furfural and HMF are both inhibitory at higher concentrations; however, concentrations below these values can enhance the performance of microorganisms, even though the growth rates decrease. According to Klinke et al.,[28] Baker's yeast is quite resistant to stress caused by furans. However, exceeding a furfural concentration of 2 g/L can result in decreased ethanol yields. *Zymomonas mobilis* are more sensitive to furfural concentrations and exceeding a value of ∼1.3 g/L reduces the ethanol yields. According to Modig et al.,[29] a furfural concentration of >1 g/L reduces the activity of enzymes (aldehyde dehydrogenase and pyruvate dehydrogenase) by 90%, thus acting as a significant stress factor for microorganisms. Furfural concentrations as low as 0.5 g/L start to reduce the activity of

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Table 5. Concentrations of By-Products

	Acetic Acid after Hydrolysis of Solids Fraction[g/L]		Acetic Acid in Filtrate [g/L]		Lactic Acid after Hydrolysis of Solids Fraction [g/L]		Lactic Acid in Filtrate [g/L]	
exp	value	std dev	value	std dev	value	std dev	value	std dev
1	0.50	0.15	1.41	0.24	0.00	0.00	0.26	0.00
2	0.53	0.11	1.30	0.08	0.00	0.00	0.26	0.00
3	0.66	0.11	2.07	0.32	0.00	0.00	0.57	0.09
4	0.58	0.00	4.06	0.38	0.00	0.00	1.55	0.68
5	0.57	0.03	3.73	0.93	0.16	0.00	1.40	0.68
6	0.51	0.07	3.86	0.10	0.00	0.00	1.40	0.41
7	0.52	0.08	3.90	0.74	0.27	0.00	1.41	0.27
8	0.52	0.03	3.78	0.77	0.16	0.00	1.36	0.27
9	0.51	0.08	4.65	0.65	0.00	0.00	1.54	0.22
10	0.46	0.05	6.05	0.06	0.26	0.00	2.31	0.58
11	0.50	0.10	5.65	0.01	0.25	0.08	1.79	0.39
12	0.43	0.06	6.66	0.45	0.25	0.00	2.02	0.56

	Furfural after hydrolysis of solids fraction [g/L]		Furfural in filtrate [g/L]		HMF after hydrolysis of solids fraction [g/L]		HMF in filtrate [g/L]	
exp	value	std dev	value	std dev	value	std dev	value	std dev
1	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.26	0.11	0.00	0.00	0.00	0.00
4	0.05	0.00	1.96	0.00	0.00	0.00	0.00	0.09
5	0.00	0.00	4.20	0.00	0.00	0.00	0.35	0.00
6	0.00	0.00	1.85	0.00	0.00	0.00	0.18	0.00
7	0.00	0.00	3.30	0.00	0.00	0.00	0.35	0.00
8	0.00	0.00	1.73	0.00	0.00	0.00	0.10	0.00
9	0.15	0.00	4.76	0.00	0.00	0.00	0.40	0.08
10	0.16	0.06	8.12	0.00	0.00	0.00	1.21	0.14
11	0.13	0.00	7.19	0.00	0.12	0.00	1.69	0.26
12	0.10	0.00	7.96	0.00	0.10	0.02	2.56	0.61

enzymes. A HMF concentration of 7 g/L reduces the ethanol yield of Baker's yeast by 50%, while HMF concentrations of <3 g/L do not reduce the ethanol yield for *Z. mobilis*.[28] However, according to Modig et al.,[29] and Liu et al.,[30] HMF is inhibitory to these enzymes, even at concentrations well below 1 g/L with 0.5 g/L as a potential inhibition threshold. It can be seen in Table 5 that the highest furfural concentration in the enzymatic hydrolyzate was found in experiment 10 (0.16 g/L), which was much lower than the potential inhibition threshold. Experiments 5−8 showed no furfural production in the enzymatic hydrolyzate. In all hydrothermal post-treatment filtrate samples obtained at temperatures of >170 °C, the production of furfural exceeds 1 g/L. There is a positive correlation between increasing temperature and furfural production. Increased furfural concentrations were also correlated with decreased xylose recoveries (see Table 4), since furfural was produced by xylose degradation.

HMF was only present in the enzymatic hydrolyzate in experiments 11 and 12, with low concentrations of 0.12 and 0.10 g/L, respectively. HMF concentrations increased in the hydrothermal post-treatment filtrate samples, with temperatures exceeding 210 °C resulting in values of >1 g/L. Therefore, the enzymatic hydrolyzate can be considered inhibitor-free; however, the filtrate should not be used in fermentation without prior detoxification. Furthermore, the amount of xylose and glucose in the filtrate is relatively low, which suggests that detoxification would not be economically feasible.

Considering all evaluated responses, the conditions of experiments 5−8 and 10 could be considered to be promising; however, economical analysis as well as trial ethanol fermentation should be performed to make a more conclusive decision.

3.3. Response Surface Analysis. Regression equations can be found below with independent variables X_1, which represents temperature (°C) and X_2, which stands for time (in minutes). The response variables are denoted as follows: Y_1 represents the glucose enzymatic hydrolysis yield, Y_2 the glucose treatment efficiency, Y_3 the xylose enzymatic hydrolysis recovery, Y_4 the xylose hydrothermal filtrate recovery, Y_5 the acetic acid concentration after enzymatic hydrolysis, Y_6 the acetic acid concentration in the hydrothermal filtrate, Y_7 the furfural concentration after the enzymatic hydrolysis, Y_8 the furfural concentration in the hydrothermal filtrate, Y_9 the HMF concentration in the hydrothermal filtrate, and Y_{10} the lactic acid concentration in the hydrothermal filtrate.

$$Y_1 = 81.73 + 16.95X_1 + 0.30X_2 - 1.857X_1X_2 - 12.82{X_1}^2 + 1.35{X_2}^2 \quad (8)$$

$$Y_2 = 85.75 + 14.12X_1 - 0.09X_2 - 1.45X_1X_2 - 10.873{X_1}^2 + 1.40{X_2}^2 \quad (9)$$

$$Y_3 = 35.76 - 18.22X_1 - 2.71X_2 - 0.97X_1X_2 - 4.764{X_1}^2 - 0.48{X_2}^2 \quad (10)$$

$$Y_4 = 15.09 - 7.31X_1 + 4.05X_2 - 7.418X_1X_2 \quad (11)$$

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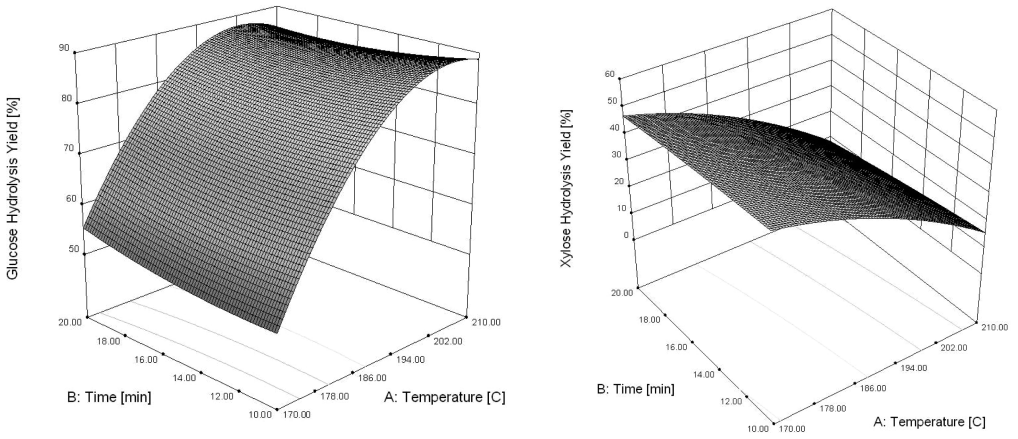

Figure 3. Response surface plots for glucose yield and xylose recovery during enzymatic hydrolysis.

$$Y_5 = 0.47 - 0.02X_1 + 0.05X_2 - 0.03X_1X_2 + 0.035{X_1}^2 + 0.06{X_2}^2 \quad (12)$$

$$Y_6 = 3.82 + 1.86X_1 - 0.11X_2 - 0.08X_1X_2 + 0.108{X_1}^2 + 0.04{X_2}^2 \quad (13)$$

$$Y_7 = 0.03 + 0.09X_1 + 0.01X_2 + 0.02X_1X_2 + 0.033{X_1}^2 + 0.03{X_2}^2 \quad (14)$$

$$Y_8 = 1.76 + 1.61X_1 + 0.21X_2 \quad (15)$$

$$Y_9 = 0.13 + 0.82X_1 + 0.12X_2 + 0.12X_1X_2 + 0.565{X_1}^2 + 0.04{X_2}^2 \quad (16)$$

$$Y_{10} = 1.39 + 0.81X_1 - 0.031X_2 - 0.21X_1X_2 - 0.25{X_1}^2 + 0.08{X_2}^2 \quad (17)$$

The correlation coefficeint (R^2) values showed that the models for each response variable explain well the relationships among the variables (all above 0.89). The analysis of variance (ANOVA) showed that the temperature at which hydrothermal treatment was performed had a significant effect (p-value of <0.0001) on both glucose yield and xylose recovery during the enzymatic hydrolysis. The time factor was much less significant than temperature in both cases. This trend was evident in the response surface plots, which are shown in Figure 3.

It can be seen from the plots that increasing the temperature had a positive effect on glucose yield in the hydrolysis up to 210 °C, where it reached its maximum and then gradually decreased (Figure 3). The decrease can be explained by glucose degradation to HMF, which becomes more favorable at temperatures above 210−220 °C.[24] Lower temperatures (below 200 °C) did not generate pH values that were low enough to initiate cleavage of the lignin−carbohydrate linkages; thus, the maximum glucose yields have been found between these two temperature ranges.[25]

In the case of xylose recovery, increasing the temperature had a negative effect—the lower the temperature, the higher the xylose recovery. Degradation to furfural can occur at temperatures of >170 °C, which has also been observed by other researchers.[25] Therefore, a balance between these two variables should be found if both products are desired. The optimization of process conditions was performed using glucose yields (both from enzymatic hydrolysis and combined treatment) as the most important response variables. Xylose yield in the hydrolysis was used as a secondary response variable for optimization. The response surface optimization resulted in optimal conditions at 191 °C and 10 min. Under these conditions, the predicted yields of glucose and xylose hydrolysis were found to be 84% and 37%, respectively.

The sequential process could be applied to other herbaceous lignocellulosic energy crops such as switchgrass, big blue stem, or miscanthus grasses, and some of the waste materials such as corn stover and sugar cane bagasse, since both steps of the process target ether bonds between lignin and carbohydrates and the amounts of these components are similar in all of those feedstocks.[31] Hydrothermal treatment has already been applied to various different feedstocks, which confirms its universality.[32−34]

3.4. Energy Recovery Efficiency. The final mass balance of the analyzed products can be found in Table 6. Samples of raw PCG (12.55 MJ/kg) and lignin extracted (22.46 MJ/kg) in the clean fractionation step were analyzed using an oxygen bomb calorimeter (Model 1341, Parr Instrument Company, Moline, IL) to measure their heating values, while the ethanol heating value (40.25 MJ/kg) was found in the *NIST Chemistry Webbook*. Heating values and mass balance were used to calculate final energy recovery efficiency (expressed by eq 18):

Table 6. Mass Balance of the Analyzed Products

	input	output	theoretical ethanol yield
overall biomass	10.54 g (9.9 gDM)		
glucose	3.63 g	3.34 g	1.71 g
xylose	1.34 g	0.78 g	0.40 g
lignin	2.08 g	0.42 g	

$$\eta = \frac{(22462 \times 0.42) + (40250 \times 1.71) + (40250 \times 0.40)}{(12547 \times 10.54)} = 0.7135 = 71.35\% \quad (18)$$

Therefore, 71.35% of the energy contained in the initial substrate was estimated to be recovered in the final products.

4. CONCLUSION

The two-step process—combined clean fractionation and hydrothermal treatment—gives the possibility of obtaining clean fractions of lignin and digestible cellulose (which also contains

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digestible hemicellulose) under nonsevere conditions without the use of mineral acids or other catalysts. The only chemicals used in this process were organic solvents (ethanol and ethyl acetate), which can be recovered by distillation. Reducing the process temperature and eliminating the catalyst from the clean fractionation step without hydrothermal treatment resulted in low cellulose digestibility (38%). Applying hydrothermal treatment after clean fractionation greatly enhanced the glucose yield (up to 90%). By-products such as acetic acid, furfural, and hydroxymethyl furfural (HMF) were found in hydrothermal filtrate. Xylose recovery from hydrothermal filtrate is difficult and costly, but removing a large part of xylose during clean fractionation prevents its loss to the hydrothermal filtrate. The hydrothermal filtrate with dissolved inhibitors contains low amounts of xylose and glucose and therefore can be discarded. Glucose yield and xylose recovery from enzymatic hydrolysis were optimized, resulting in the highest glucose (92%) and xylose (58%) yields.

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Notes

The authors declare no competing financial interest.

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