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Catalyzed modified clean fractionation of prairie cordgrass integrated with hydrothermal post-treatment

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ABSTRACT

The main purpose of this study was pretreatment of prairie cordgrass obtaining three fractions: lignin, hemicellulose and fermentable cellulose. Modified clean fractionation process (using ethyl acetate–ethanol–water mixture) was employed to extract lignin and hemicellulose fractions. Different proportions of constituents in the solvent mixture were tested. In order to improve lignin recovery, the process was catalyzed with sulfuric acid. Optimization was performed for fractionation processing conditions using lignin recovery and glucose yields as primary response variables. Optimal conditions resulted in a 51.33% lignin recovery, 53.82% hydrolysis glucose yield and 33.73% xylose yield in the aqueous fraction. To improve cellulose digestibility, solid fraction was subjected to hydrothermal post-treatment, for which optimal processing conditions were found, resulting in 78.93% hydrolysis glucose yield. This research represents a step forward for biomass pretreatment, and may open up new possibilities for utilization of prairie cordgrass.

1. Introduction

Lignocellulose is the most abundant and accessible renewable biomass in the world. As an alternative to petroleum, it has great potential for being a source of energy and value-added products [1]. Containing cellulose, hemicellulose and lignin in its structure, lignocellulose is a complex source of carbohydrates and various organic (mostly phenolic) compounds. In recent years, it has become popular as a substrate for the production of fuel ethanol but in order to fully utilize its potential, it must be fractionated prior to further processing. Furthermore, an efficient pretreatment method is necessary to obtain fermentable sugars and thus achieve high ethanol yield. Connecting these two main goals (biomass fractionation and pretreatment) can facilitate the implementation of the complete biorefinery concept.

Herbaceous plants (i.e., grasses and other agricultural residues) are good potential ethanol feedstocks due to high cellulose content, its biochemical accessibility, and potential for sustainability. Prairie cordgrass is an abundant grass species, especially common to southwestern and southeastern parts of the U.S., the upper Great Plains, and Canada. It contains about 40% cellulose, 20% lignin, and up to 30% hemicellulose. It is an especially attractive substrate for ethanol production because of high cellulose content. It is a non-food biomass, and it is too coarse to be used as animal feed [2,3].

Production of ethanol from lignocellulosic biomass requires efficient pretreatment. Organosolv treatment is a method that can be used as simultaneous biomass pretreatment and fractionation, since it removes lignin from the lignocellulose, and makes the cellulose more digestible at

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the same time [1]. The principle of organosolv treatment is dissolving the lignin component in an organic solvent (e.g. ethanol, acetic acid, acetone, methyl isobutyl ketone), while the cellulose fraction remains in the solid [4]. In some processes, water is added to the solution in order to extract hemicellulose components and by-products, and to obtain higher purity of the desired cellulose and lignin streams [5,6]. Organosolv lignin is easily recoverable and relatively pure (i.e., low in ash and carbohydrate contamination), which makes it an attractive source of phenolic compounds which can be used to produce resins, chemicals, or pharmaceuticals [7–9]. The hemicellulose fraction can be utilized for recovery of xylose, which constitutes a significant portion of the lignocellulose composition.

The organosolv treatment process originated from the pulp and paper industry. Developed processes (ALCELL, Lignol, Biodyne) result in production of cellulose pulp (which can be used for ethanol production) and lignin (which has been found to be relatively free of contaminants and highly phenolic) [4,5,10]. One type of organosolv treatment is Clean Fractionation, developed by the National Renewable Energy Laboratory in 1994 [6]. This process was based on the use of a mixture of methyl isobutyl ketone (MIBK), ethanol and water. It has been successfully applied to softwoods and sugar cane bagasse [6], and recently to prairie cordgrass [11]. However, MIBK costs and toxicity have made the process economically unfeasible, so the research was not taken beyond a small pilot-scale (semi-continuous mode) [6]. Organosolv treatment provides a unique opportunity for biomass fractionation, because it creates three separate streams (cellulose, hemicellulose and lignin rich streams), each with different downstream processing possibilities.

In order to achieve high lignin recovery, and maintain processing temperatures below 180 °C, catalyst use is often necessary [12]. Catalysts are oftentimes used in organosolv treatments in order to initiate ether bonds cleavage (between lignin and carbohydrates) at lower temperatures, and therefore decrease required processing temperatures. Effective catalysts include sulfuric acid, phosphoric acid, magnesium chloride and sodium bisulfate [13,14].

Combining pretreatment methods can lower the harshness of individual processes and achieve maximum yields of the desired end products. Integrated processes can exhibit better selectiveness and result in streams of higher purity than a single treatment alone [15]. Examples include alkali- or acid-based treatments combined with each other [16], with steam explosion [17] or with organosolv treatment [18]. Hydrothermal treatment has been found to be efficient in enhancing enzymatic cellulose digestibility in prairie cordgrass [19], so its application as a post-treatment after clean fractionation may give better glucose yields during enzymatic hydrolysis, than clean fractionation alone.

Hydrothermal treatment (also referred to as autohydrolysis) does not involve use of any chemicals. It is based on carbohydrates–lignin bonds cleavage by hydronium ions first generated by heating water to high temperatures (150–230 °C), and subsequently released from hemicellulose hydrolysis and deacetylation [20]. The hydrothermal treatment has been successfully applied to different feedstocks for many years [21].

Combination of steam explosion with subsequent organosolv treatment has been applied to softwood with good outcomes [22]. However, as suggested in [20], lignin repolymerization occurring after hydrothermal treatment alters its structure and properties, and makes subsequent extraction less efficient. Therefore, in this study, the route of organosolv treatment followed by hydrothermal post-treatment was chosen to maximize process streams purity and applicability.

The main objectives of this study were to find optimal processing conditions for clean fractionation and hydrothermal post-treatment using two main response variables: lignin recovery (in clean fractionation) and glucose yield from enzymatic hydrolysis of the cellulose-rich solid fraction (in both steps). Pretreatment efficiency was determined by glucose yield, lignin recovery, xylose yield and ethanol yield.

2. Materials and methods

2.1. Prairie cordgrass (PCG)

Prairie cordgrass (*Spartina pectinata*) was used as a feedstock in this study. It was harvested July 1st 2007 in Yankton County, South Dakota. The specific geographical coordinates are 43° 08′ 45.49″ N 97° 10′ 00.26″ W, with the elevation of 426.72 m. The plant was in late vegetative to pre-boot stage and about 1–1.2 m tall at the time of harvest. Collected samples included entire vegetative portion of the plant. The plant material was stored in round bale form, in an enclosed structure. The entire bale was ground in a hammer mill using a 2 cm screen and the material was stored in large container bags until needed for this research project. Prior to processing, the feedstock was air dried (having final dry matter content of 93.99 ± 0.02%) and ground to pass through 1-mm screen using a Thomas-Wiley Laboratory Mill (Model 3375-E15, Thomas Scientific, USA). Composition of the material was measured using a standard method (sulfuric acid hydrolysis) according to [23], and the results (calculated as dry matter mass fraction) were as follows: 36.70 ± 0.01% glucan, 13.52 ± 2.00% xylan, 1.59 ± 0.57% arabinan, 1.40 ± 0.5% galactan, 0.30 ± 0.00% mannan 20.96 ± 0.52% lignin, and 5.65 ± 0.04% ash. Xylan constitutes ~80% of the hemicellulose, and thus xylose monitoring was chosen to represent hemicellulose distribution into all the pretreatment streams.

2.2. Experimental design

The pretreatment process was comprised of two main steps: 1) clean fractionation into three main streams (solid retentate, organic permeate, and aqueous permeate), and 2) hydrothermal post-treatment of the solid retentate from clean fractionation. After the combined pretreatment, solid fractions were subjected to enzymatic hydrolysis and fermentation. An experimental design was developed based on a central composite design (CCD) using an α value of 1.681 for clean fractionation, and 1.414 for the hydrothermal post-treatment. Both α values were chosen to ensure rotatability of the designs [24]. Experimental designs can be found in Tables 1 and 2. Experimental designs, statistical analyses and optimizations were performed using statistical software [25]. The goal of the study was also to minimize the process harshness (i.e., minimize temperature and catalyst

Table 1 – Four factor rotatable experimental design with central composite points for the clean fractionation optimization.

Experiment no.	Temperature [°C]	Coded value	Sulfuric acid (catalyst) concentration [%]	Coded value	Ethyl acetate concentration [%]	Coded value	Ethanol concentration [%]	Coded value
1	140.00	+1.00	0.46	+1.00	50.00	+1.00	10.00	−1.00
2	140.00	+1.00	0.46	+1.00	15.00	−1.00	10.00	−1.00
3	140.00	+1.00	0.15	−1.00	50.00	+1.00	35.00	+1.00
4	110.00	−1.00	0.46	+1.00	15.00	−1.00	35.00	+1.00
5	140.00	+1.00	0.15	−1.00	15.00	−1.00	35.00	+1.00
6	110.00	−1.00	0.15	−1.00	50.00	+1.00	10.00	−1.00
7	110.00	−1.00	0.46	+1.00	50.00	+1.00	35.00	+1.00
8	110.00	−1.00	0.15	−1.00	15.00	−1.00	10.00	−1.00
9	99.77	−1.68	0.31	0.00	32.50	0.00	22.50	0.00
10	150.23	+1.68	0.31	0.00	32.50	0.00	22.50	0.00
11	125.00	0.00	0.04	−1.68	32.50	0.00	22.50	0.00
12	125.00	0.00	0.57	+1.68	32.50	0.00	22.50	0.00
13	125.00	0.00	0.31	0.00	3.07	−1.68	22.50	0.00
14	125.00	0.00	0.31	0.00	61.93	+1.68	22.50	0.00
15	125.00	0.00	0.31	0.00	32.50	0.00	1.48	−1.68
16	125.00	0.00	0.31	0.00	32.50	0.00	43.52	+1.68
17	125.00	0.00	0.31	0.00	32.50	0.00	22.50	0.00
18	125.00	0.00	0.31	0.00	32.50	0.00	22.50	0.00
19	125.00	0.00	0.31	0.00	32.50	0.00	22.50	0.00
20	125.00	0.00	0.31	0.00	32.50	0.00	22.50	0.00

concentration). The maximum temperature (140 °C) and catalyst concentration (0.57%) were chosen based on the NREL clean fractionation optimal conditions [6]. Minimum temperature was chosen based on the standard boiling point of the highest boiling ingredient of the solvent (i.e., water), and was thus equal to ~100 °C. Minimum catalyst concentration (sulfuric acid was used in this study) was chosen to ensure that low star point for this factor had a positive value (0.04%). Catalyst concentrations were expressed as mass fraction based on the solvent mixture total mass (weight of catalyst/weight of solvent–catalyst mixture). Ethyl acetate and ethanol content ranges in the solvent were chosen to ensure maintaining a one phase mixture at room temperature (based on the binodal phase diagram [26]). Fractionation solvent mixture components were expressed as solvent mixture mass fraction (weight of specific component of the solvent mixture/total weight of the solvent–catalyst mixture). All design points (factorial, axial and center) were performed in duplicate.

Table 2 – Two factor rotatable experimental design with central composite points for the hydrothermal post-treatment optimization.

Experiment no.	Temperature [°C]	Coded value	Time [min]	Coded value
1	170.00	−1.00	10.00	−1.00
2	210.00	+1.00	10.00	−1.00
3	170.00	−1.00	20.00	+1.00
4	210.00	+1.00	20.00	+1.00
5	161.72	−1.41	15.00	0.00
6	218.28	+1.41	15.00	0.00
7	190.00	0.00	7.93	−1.41
8	190.00	0.00	22.07	+1.41
9	190.00	0.00	15.00	0.00
10	190.00	0.00	15.00	0.00
11	190.00	0.00	15.00	0.00
12	190.00	0.00	15.00	0.00

2.3. *Catalyzed modified clean fractionation*

Both pretreatment processes (clean fractionation and hydrothermal post-treatment) were performed in custom-made 250 mL capacity reactor tubes, fitted in an aluminum heating block (holding 6 tubes at a time). The heating block temperature was controlled by auto-tune temperature controllers (CN9121A, Omega). The reactors were equipped with thermocouples attached to the reactor screw caps. The temperature profile in each reactor was monitored by Lab View software (version 8.2, National Instruments, Austin, TX) utilizing a data acquisition device (OMB-DAQ-56, Omega). Pressure was monitored with pressure gauges attached to the screw caps of the reactors.

The two-step pretreatment process began with biomass clean fractionation, using a three-component solvent mixture with the addition of sulfuric acid (72% stock solution, Fisher Scientific) as a catalyst. The fractionation solvent mixture was modified from the original NREL clean fractionation solvent mixture, and was composed of ethyl acetate, ethanol and water, mixed in different proportions (Table 1). Ethyl acetate was selected to replace MIBK (which was used in the original NREL clean fractionation), due to its lower toxicity and decreased cost. Experimental factors (Table 1) included temperature, solvent composition (ethanol and ethyl acetate content), and catalyst concentration. Solvent constituents' proportions and catalyst concentration were presented as mass fractions of the entire biomass–solvent–catalyst mixture. Digestion time was found to be a non-significant factor by preliminary trials (data not shown). Digestion time was also found to be non-significant in previous study performed on a non-catalyzed integrated process of clean

fractionation and hydrothermal post-treatment applied to the same feedstock [12]. Therefore, in this study, digestion time was kept constant and equal to 20 min throughout all experiments. The zero time was taken when the system reached the pre-set desired temperature.

Biomass loading was constant and equal to 10% of the dry matter in the biomass–solvent–catalyst mixture (mass fraction); dry matter content of the raw prairie cordgrass was determined to be 93.99 ± 0.11%. Totally 100 g of the biomass–solvent–catalyst mixture was placed in the reactor. Preheating time was in the range of 20–40 min, and cooling time (by water bath) was 20 min. After the reaction, the post-reaction slurry was filtered through a polypropylene cloth (permeability factor of 708 L min^{-1}) by vacuum filtration. The solid residue was washed with 40 mL of ethyl acetate and 100 mL of water, in order to reduce lignin condensation on the cellulose fibers and to initiate phase separation of the liquid permeate. The solid retentate fraction was then subjected to enzymatic hydrolysis, after which the glucose released to the enzymatic hydrolyzate was measured on a High Performance Liquid Chromatography system (Agilent HPLC 1200 Series, Santa Clara, CA, USA) [27,28]. The liquid permeate fraction was then separated into organic and aqueous phases; the organic stream contained mainly organic constituents and dissolved lignin, while the aqueous stream contained mainly water and small quantities of organic components, by-products and dissolved sugars. The organic fraction was then evaporated until dry, and lignin recovery was evaluated based on the mass of the remaining solid. The aqueous fraction was analyzed on a High Performance Liquid Chromatography system (Agilent HPLC 1200 Series, Santa Clara, CA, USA) for dissolved sugars and generated by-products according to [27].

The experimental optimization results were then validated by applying optimal processing conditions to additional raw biomass samples with four replications, following the same procedures which were used during the optimization experiments.

2.4. Hydrothermal post-treatment

In order to improve cellulose enzymatic digestibility, and thus increase the glucose yield, the solid fraction obtained from the optimal clean fractionation conditions was subjected to hydrothermal post-treatment. This treatment was conducted in the same reactor tube set-up, using 50 g total weight of deionized water–biomass mixture, with 10% (mass fraction) of dry matter loading of solid retentate obtained from fractionation process. Clean fractionated solid retentate dry matter content was determined to be 23.24 ± 0.79%. Optimization experiments were performed for two factors: temperature and processing time (Table 2). No catalyst was added to the reaction mixture at this step.

Pre-heating time varied from 20 to 40 min, while water-bath cooling time was about 20 min. After the reaction, the slurry was filtered through the polypropylene cloth by vacuum filtration and the solid retentate was washed with 50 mL of deionized water. The obtained solid was then subjected to enzymatic hydrolysis in order to measure cellulose digestibility (by glucose yield). The filtrate was analyzed for dissolved sugars and generated by-products according to [27].

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was performed after each step of the combined process: first, on the solid fraction obtained from clean fractionation, and second, on the solid fraction obtained from hydrothermal post-treatment. The hydrolysis was performed according to [28], in 20 mL scintillation vials, placed in a shaking-bed incubator (Incubated Tabletop Orbital Shaker Model 420, Fisher Thermo Scientific), with revolution frequency 2.5 Hz and dry matter loading of 3% (as mass fraction of the entire mixture), at pH 4.8. Temperature was kept constant at 50 °C, and the hydrolysis duration was 72 h. Enzymes included cellulase (Novozymes, NS50013) and β–glucosidase (Novozymes, NS50010), added in amounts 15 FPU g^{-1} dry matter and 60 CBU g^{-1} dry matter, respectively. Released sugars and by-products concentrations in hydrolyzates were analyzed on HPLC instrument (Agilent HPLC 1200 Series, Santa Clara, CA, USA) according to [27].

2.6. Simultaneous saccharification and fermentation (SSF)

After finding optimal conditions for both clean fractionation and hydrothermal post-treatment, SSF was performed on the optimally-treated samples. This was done in order to evaluate the performance of *Saccharomyces cerevisiae* on the pretreated samples, and therefore to measure resulting practical ethanol yield. This procedure was based on NREL protocols [27,29]. *S. cerevisiae* D_5A (no. 200062) was obtained from ATCC (Manassas, VA, USA).

The *S. cerevisiae* yeast culture was grown on a solid medium (composed of sterile agar, peptone, and glucose) and refrigerated until used. Growth medium for the SSF was prepared in 250 mL flasks out of glucose, yeast extract, and deionized water. Glucose-to-yeast extract ratio was 10:1. The growth medium was sterilized by autoclaving directly after preparation. Inoculation was initiated by inserting a yeast culture scratched from the agar plate into the growth medium and incubating at 35 °C and revolution frequency of 4.17 Hz for 24 h prior to SSF start-up.

The SSF starting mixture was prepared in the same manner as for enzymatic hydrolysis, according to [29]; however a 100 mL volume was used (in 250 mL flasks with glycerol-filled yeast locks). Biomass loading was kept at 3% dry matter, pH at 4.8, and enzymes were added in amounts of 15 FPU g^{-1} dry matter of cellulase and 60 CBU g^{-1} dry matter of β–glucosidase. Yeast extract was added in an amount of 0.5%, and yeast culture in the amount of 1% of the total volume. Tetracycline (added in an amount of 0.3%) was used as an antibiotic to avoid contamination with *lactobacilli* and undesired production of lactic acid. Enzymes and yeast were added to the mixture last.

The SSF was then performed for 168 h (7 days), at 35 °C, and revolution frequency of 1.5 Hz, in triplicate. Samples were collected aseptically after 0, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168 h. Ethanol produced, remaining glucose, as well as by-product concentrations were measured by HPLC system (Agilent HPLC 1200 Series, Santa Clara, CA, USA).

2.7. Glucose yield

Hydrolysis and total glucose yields were calculated based on the concentrations found after the completion of the enzymatic hydrolysis reaction compared to the glucose amount in

the solids after the pretreatment and glucose amount in raw biomass. The hydrolysis yield was calculated by Eq. (1), which is solely a calculation of cellulose enzymatic digestibility after a given treatment (equivalent to the efficiency of cellulose to glucose conversion).

$$\text{Hydrolysis glucose yield [\%]} = \frac{\text{Glucose amount after hydrolysis [g]}}{\text{Glucose amount in pretreated material [g]}} \times 100\% \quad (1)$$

Total glucose yield was calculated by comparing the glucose amount released during the enzymatic hydrolysis to the amount of glucose in the raw biomass, which was analyzed by acid hydrolysis [23]. Eq. (2) shows the total glucose yield, which accounts for losses of glucose dissolved in the liquid fractions.

$$\text{Total glucose yield [\%]} = \frac{\text{Glucose amount after hydrolysis [g]}}{\text{Glucose amount in raw material [g]}} \times 100\% \quad (2)$$

2.8. Lignin recovery

Clean fractionation (CF) lignin recovery was calculated directly from mass balance, based on the lignin content in the raw biomass compared to the solids remaining after the organic fraction drying. Eq. (3) summarizes this relationship.

$$\text{Lignin recovery [\%]} = \frac{\text{Lignin amount after CF [g]}}{\text{Lignin amount in raw material [g]}} \times 100\% \quad (3)$$

2.9. Xylose yield

In addition to glucose and lignin yields (the response variables used for optimization), xylose yields were calculated to evaluate hemicellulose removal and degradation (Eqs. (4)–(6)). Xylose concentrations were measured in the enzymatic hydrolyzates, in the aqueous fractions from clean fractionation, and in the filtrates from hydrothermal post-treatment.

$$\text{Hydrolysis xylose yield [\%]} = \frac{\text{Xylose amount after hydrolysis [g]}}{\text{Xylose amount in raw material [g]}} \times 100\% \quad (4)$$

$$\text{Aqueous fraction xylose yield [\%]} = \frac{\text{Xylose amount in the aqueous fraction [g]}}{\text{Xylose amount in raw material [g]}} \times 100\% \quad (5)$$

$$\text{Hydrothermal filtrate xylose yield [\%]} = \frac{\text{Xylose amount in the hydrothermal filtrate [g]}}{\text{Xylose amount in raw material [g]}} \times 100\% \quad (6)$$

2.10. Ethanol yield

Theoretical ethanol concentration was calculated using the glucan content in the solids and fermentation equation stoichiometry (Eqs (7) and (8)). The relative ethanol yield or fermentation efficiency was calculated as a ratio of the actual ethanol concentration at the end of the SSF process to the theoretical concentration (Eq. (9)). Since tetracycline shows a peak overlapping with the ethanol peak on the HPLC chromatogram, initial artificial ethanol concentration was subtracted from the final ethanol concentration to avoid false results.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (7)$$

$$\text{Theoretical ethanol concentration } [\text{g L}^{-1}] = 0.51 \times \text{Glucan content in the solids } [\text{g L}^{-1}] \quad (8)$$

$$\text{Relative ethanol yield [\%]} = \frac{\text{Final ethanol concentration} - \text{Initial artificial ethanol concentration}}{\text{Theoretical ethanol concentration}} \times 100\% \quad (9)$$

3. Results and discussion

The response variables for optimization of clean fractionation were lignin recovery and glucose yields after enzymatic hydrolysis, while for hydrothermal post-treatment response variables included hydrothermal and total glucose yields. Additionally, xylose yields and by-product concentrations were considered for both steps.

3.1. Clean fractionation optimization

The processing conditions of clean fractionation had a significant influence on the lignin recovery, glucose and xylose yields as well as by-product generation (Table 3). The highest lignin recovery, 46.3% (experiment 10), was found at the high factorial star point (150 °C) of temperature, but at the center points of all other factors. This result was over twice as high as in uncatalyzed clean fractionation, which achieved only 20% lignin recovery [12].

The highest glucose hydrolysis yield (55.8%) and total glucose yield (50.0%) were achieved (Table 3) by the samples treated at the high factorial points of catalyst concentration (0.46%) and temperature (140 °C), but at low factorial points of ethyl acetate (15%) and ethanol (10%) contents, respectively (i.e., experiment 2). These results are also higher than in the case of the uncatalyzed clean fractionation process [12]. Hydrolysis glucose yields were found to be low when comparing to the most efficient pretreatment processes, for which glucose yields can achieve 70–100% [30–33]. It can be deducted that the reason for the low glucose yield obtained in this research was cellulose crystallinity and remaining hemicellulose components and lignin still bonded with cellulose, which were not removed by the clean fractionation pretreatment. As reported in a similar study on clean fractionation of prairie cordgrass, low lignin and xylan removal resulted in low enzymatic hydrolysis glucose yields and cellulose digestibilities. When xylan and lignin removal was lower than 50% (% of initial xylan and lignin content in raw

Table 3 – Results obtained from clean fractionation process (presented with ±1 standard deviation).

Exp.	Lignin recovery [%]	Hydrolysis glucose yield [%]	Total glucose yield [%]	Xylose aqueous fraction yield [%]	Hydrolysis xylose yield [%]	Acetic acid concentration in the hydrolyzate [g L^{-1}]	Acetic acid concentration in the aqueous fraction [g L^{-1}]
1	39.53 ± 2.61	53.47 ± 0.36	49.39 ± 0.07	36.92 ± 3.36	27.28 ± 0.25	0.28 ± 0.01	13.75 ± 0.77
2	10.48 ± 1.33	55.82 ± 0.98	49.99 ± 1.12	55.98 ± 6.72	20.85 ± 3.81	0.19 ± 0.05	16.73 ± 3.61
3	26.95 ± 9.11	31.61 ± 1.11	29.75 ± 1.16	28.58 ± 2.14	18.54 ± 4.55	0.15 ± 0.05	3.71 ± 1.09
4	10.97 ± 0.00	29.54 ± 0.76	26.93 ± 0.65	28.92 ± 0.49	11.90 ± 0.31	0.09 ± 0.01	8.23 ± 0.74
5	12.41 ± 1.36	34.23 ± 2.00	31.02 ± 1.80	32.08 ± 0.02	14.87 ± 2.21	0.13 ± 0.02	0.00 ± 0.00
6	10.98 ± 0.00	30.32 ± 4.65	27.46 ± 3.71	29.35 ± 3.11	12.77 ± 5.20	0.36 ± 0.39	8.39 ± 0.00
7	40.08 ± 4.70	41.97 ± 7.53	38.69 ± 6.39	30.80 ± 6.02	41.77 ± 0.00	0.16 ± 0.00	13.70 ± 0.67
8	3.34 ± 2.03	27.46 ± 1.89	24.71 ± 1.70	29.61 ± 0.16	10.24 ± 2.74	0.16 ± 0.11	0.04 ± 0.06
9	17.18 ± 0.69	27.89 ± 0.16	25.47 ± 0.28	28.21 ± 3.80	9.75 ± 0.79	0.09 ± 0.00	11.35 ± 0.00
10	46.31 ± 0.00	47.79 ± 0.00	44.13 ± 0.00	28.08 ± 0.00	34.64 ± 0.00	0.31 ± 0.00	8.17 ± 0.00
11	13.82 ± 1.36	29.05 ± 0.41	26.07 ± 0.30	17.67 ± 0.76	10.38 ± 0.02	0.04 ± 0.06	0.00 ± 0.00
12	37.20 ± 2.02	51.41 ± 1.59	47.32 ± 2.08	36.77 ± 5.10	33.10 ± 2.69	0.27 ± 0.06	28.59 ± 0.44
13	4.05 ± 1.69	32.50 ± 3.67	29.43 ± 3.27	31.64 ± 1.88	15.05 ± 4.29	0.06 ± 0.08	1.52 ± 0.80
14	40.75 ± 3.63	39.48 ± 1.47	36.64 ± 1.31	32.75 ± 0.03	28.77 ± 4.41	0.22 ± 0.03	12.36 ± 2.70
15	7.39 ± 1.03	33.09 ± 1.90	30.01 ± 1.63	32.56 ± 1.24	15.17 ± 2.50	0.12 ± 0.01	3.72 ± 2.07
16	16.66 ± 1.32	35.12 ± 3.08	32.66 ± 3.96	33.05 ± 0.00	23.78 ± 6.23	0.17 ± 0.03	5.72 ± 2.25
17	29.54 ± 4.00	35.89 ± 2.69	32.90 ± 2.52	28.77 ± 0.67	18.97 ± 5.44	0.14 ± 0.05	11.73 ± 0.00
18	31.00 ± 3.41	37.72 ± 0.72	34.54 ± 0.69	29.05 ± 0.01	22.45 ± 1.90	0.18 ± 0.00	9.30 ± 3.02
19	27.59 ± 4.07	37.55 ± 1.37	34.44 ± 1.13	28.09 ± 0.68	20.46 ± 1.27	0.17 ± 0.03	7.16 ± 1.38
20	30.45 ± 3.38	38.51 ± 2.81	35.38 ± 2.71	28.28 ± 0.99	21.89 ± 5.36	0.16 ± 0.03	6.74 ± 3.14

PCG) total enzymatic hydrolysis glucose yield did not exceed 50%, whereas enzymatic hydrolysis efficiency did not exceed 60% [11]. Glucose concentration in aqueous fractions was very low, achieving the highest value of ~7% of the input glucose (occurring at the center points for all factors). It is desired to keep the glucose concentration in the liquid fraction low, since it reduces glucose yield in the solid fraction.

Xylose aqueous fraction yield showed a positive correlation ($R^2 = 0.686$) with hydrolysis glucose yield (Fig. 1A). This suggests that xylose removal into the aqueous fraction influenced cellulose digestibility. Strong relationship between xylose removal and cellulose digestibility ($R^2 = 0.960$) was observed in previous work of clean fractionation of PCG using MIBK in the solvent mixture [11]. Xylose extracted to the

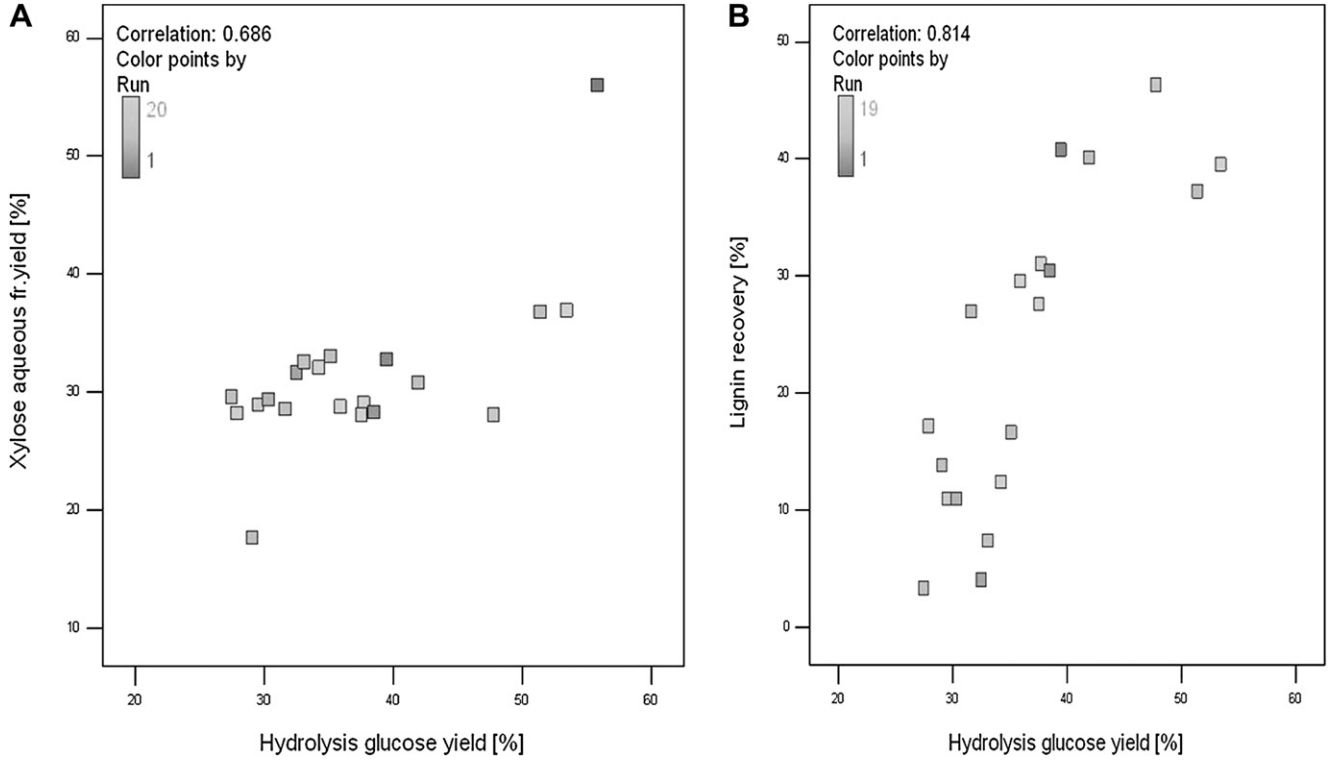

Fig. 1 – Correlations between lignin/xylose recovery and hydrolysis glucose yield.

aqueous fraction could be recovered and used in chemical production (e.g. xylitol). As was anticipated, lignin removal (represented by the lignin recovery variable) was also found to have a positive correlation (0.814) with hydrolysis glucose yield (Fig. 1B), which confirms the hypothesis that delignification increases cellulose digestibility. Similar trend ($R^2 = 0.950$) was observed in case of clean fractionation of PCG using MIBK in the solvent mixture [11]. Xylose remaining (Table 3) in the solid fraction was hydrolyzed during the enzymatic hydrolysis, however giving low yields (9.75%–41.77%). Xylose released in the enzymatic hydrolysis could be used in the co-fermentation process with glucose, using genetically modified yeast or naturally occurring microorganisms, but none of these processes have yet been proven to be effective on a commercial scale to date [33,34].

The only by-product generated in the clean fractionation process (and mostly filtrated into the aqueous fraction) was acetic acid. Acetic acid concentration in the enzymatic hydrolyzate did not exceed the stress level for yeast (0.5 g L^{-1}) [35]. No furfural or hydroxymethyl furfural (HMF) was detected in any of the fractions either. Acetic acid was mainly generated not due to hemicellulose deacetylation, but due to ethyl acetate acid-catalyzed hydrolysis with co-generation of ethanol.

The statistical optimization of temperature, sulfuric acid (catalyst) concentration, ethyl acetate and ethanol content was performed using three response variables: lignin recovery, hydrolysis glucose yield and total glucose yield. The regression models and response surface plots which were developed for these response variables, as well as for xylose yields, can be found in Fig. 2A–C.

In the optimization step, lignin recovery was used as the primary response variable, while glucose yields were secondary variables. The highest lignin recovery followed by the highest glucose yields were the main goals of the optimization. Numerical optimization methods provided several solutions for specified optimization conditions. Only one set of conditions was chosen, considering the most desirable results and possibly the least harsh conditions (i.e., lowest temperature and lowest concentrations of chemicals). Based on the numerical optimization results, the optimal conditions were found at 139.71 °C, 0.39% catalyst concentration, 36.87% ethyl acetate content and 25.49% ethanol content. At these conditions, lignin recovery was predicted to achieve a result of 46.49%, hydrolysis glucose yield was predicted to be 49.40% and predicted total glucose yield was 46.20%. Predicted xylose aqueous fraction yield was found to be 33.37%, while predicted hydrolysis xylose yield was 34.49%.

A model was also developed for acetic acid concentration in the aqueous fraction, since it reflected the extent of ethyl acetate hydrolysis. Catalyst (sulfuric acid) concentration was the factor which mainly influenced concentration of produced acetic acid. The trend can be clearly observed in Fig. 3. This suggests that hydronium ions presence induced ethyl acetate hydrolysis to ethanol and acetic acid [36].

Response surface graphs (Fig. 2) did not show distinct optima or maximum values; however, a plateau could be observed in the lignin recovery model. Achieving higher results for glucose yields would be associated with increasing the temperature and catalyst concentration, which means using harsher conditions when compared to NREL's clean fractionation [6], and is contradictory with the purpose of this study. Therefore, an alternative approach has been proposed, which is application of a hydrothermal post-treatment.

3.2. *Clean fractionation validation*

The results of the optimization were validated in order to confirm the accuracy of the models when applied to future samples. Therefore, the optimal processing conditions were applied to raw biomass samples, and lignin recovery along with glucose yields were measured and compared with predicted values. Additionally, xylose yields and acetic acid concentration were measured in both aqueous fractions and hydrolyzates for the validation samples. The validation analysis showed that the models developed for the response variables can be successfully used for future samples, since lignin recovery results (51.33 ± 6.24%) were within the prediction interval (41.24–51.70%), hydrolysis glucose yield (53.82 ± 1.23%) was contained within the tolerance interval (41.60–56.18%). Total glucose yield (49.96 ± 0.98%) was also within the tolerance interval (39.23–52.75%), while xylose aqueous fraction yield (33.73 ± 3.01%) and hydrolysis xylose yield (35.20 ± 0.97%) were contained in the prediction intervals (32.69–43.56% and 28.18–36.93%, respectively). Acetic acid can act as a stress factor for yeast when present in concentrations higher than 0.5 g L^{-1}. Acetic acid concentration in the enzymatic hydrolyzate was low (0.26 ± 0.02 g L^{-1}), while the aqueous fraction contained high amounts of this by-product (27.12 ± 4.85 g L^{-1}).

Lignin extracted at optimal conditions was analyzed for the Klason lignin and ash content by sulfuric acid hydrolysis [23]. The results showed that the extracted organosolv lignin contains 65.85 ± 1.56% of acid insoluble (Klason) lignin, and 2.93 ± 0.11% ash.

3.3. *Hydrothermal post-treatment optimization*

After concluding that the clean fractionation solid fraction hydrolysis glucose yield was found to be lower than expected, an enhancement procedure was used. Hydrothermal post-treatment was applied to the clean fractionation solid fraction in order to improve cellulose digestibility and increase glucose yield (Table 4). Glucose yields after the enzymatic hydrolysis of hydrothermally post-treated solid fractions were found to be higher when compared to hydrolyzed fractionated solid with no post-treatment (Table 3), achieving a maximum of 81.28%, with a total glucose yield of 74.93%. These results were achieved using moderate conditions of 190 °C with 15 min of processing time. In general, it was observed that the glucose yields, and therefore cellulose digestibility, were significantly enhanced by application of the hydrothermal post-treatment. In the case of the highest glucose yields, the results were improved by ~60% when compared to the samples treated at optimal conditions of clean fractionation alone, which thus justifies application of the post-treatment.

Xylose yields and by-product concentrations were also analyzed (Table 4). Low amounts of acetic acid and furfural were present in the enzymatic hydrolyzates, and only slightly higher concentrations (although exceeding potential stress levels for yeast) were observed in the filtrates. HMF was found

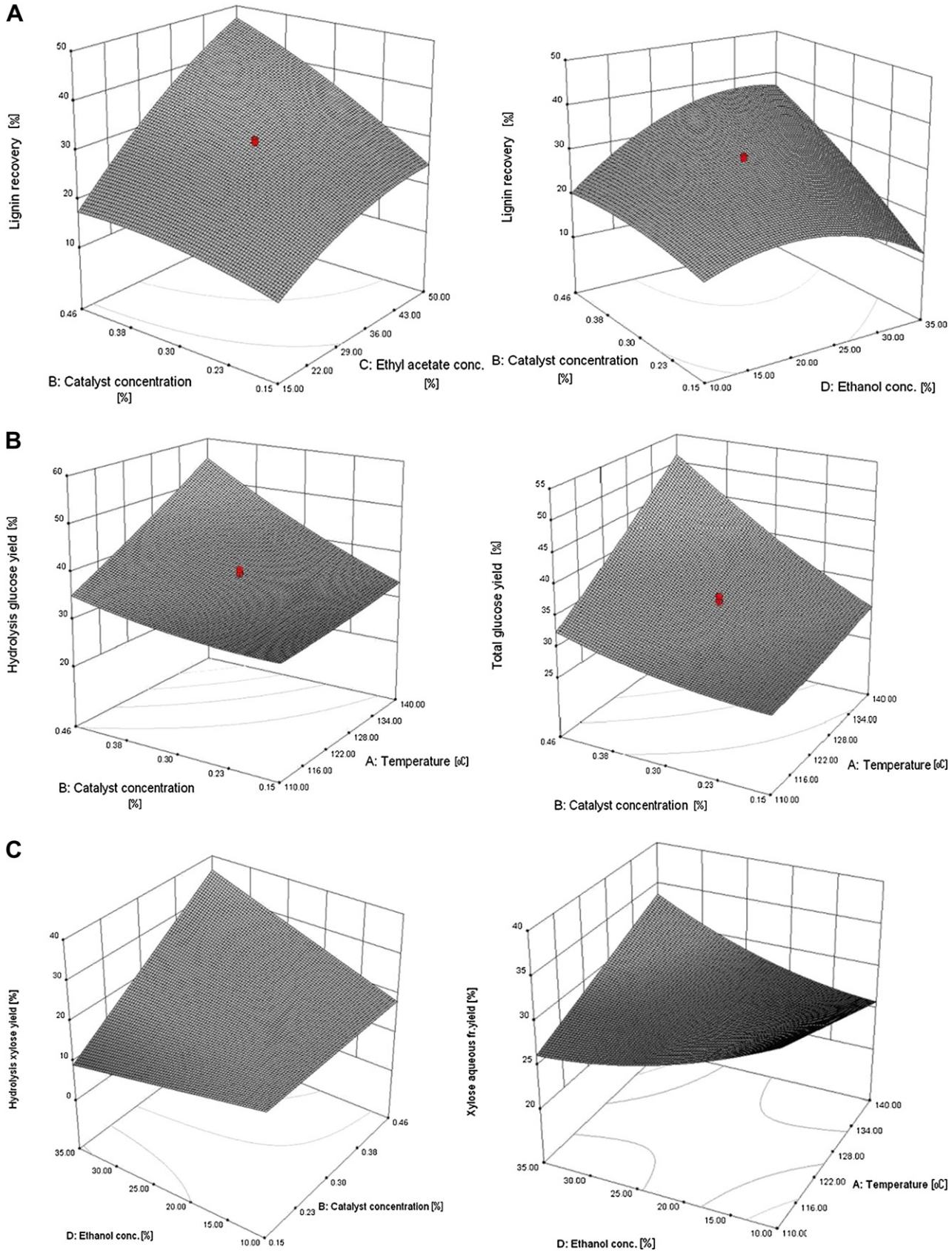

Fig. 2 – Response surface graphs for response variables [lignin, %; hydrolysis and total glucose yields, %; and hydrolysis and aqueous fraction xylose yields, %] vs. significant factors [catalyst concentration, %; ethyl acetate concentration, %; ethanol concentration, %; temperature, °C] after clean fractionation process.

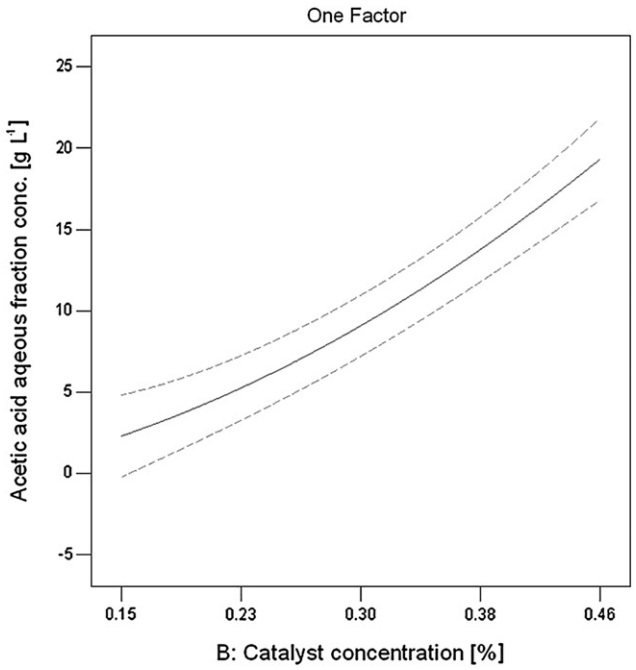

Fig. 3 – Catalyst concentration influence on acetic acid production and release into the aqueous fraction (dashed lines show the confidence intervals (95%).

in trace amounts in the enzymatic hydrolyzates, but in noticeable concentrations in the filtrates. Furfural is considered a potential stress factor for microorganisms in concentrations higher than 0.5 g L^{-1}, while HMF can lower microorganisms' performance when present in concentrations higher than 0.15 g L^{-1} [35,37].

Optimization of the hydrothermal post-treatment was then performed using both glucose yields (i.e., enzymatic hydrolysis and total) as response variables as functions of temperature and time (Fig. 4A). Additionally, models for xylose yields in the enzymatic hydrolysis and filtrate were developed (Fig. 4B). The optimum point for both glucose yields was achieved within chosen optimization operability region, and can be seen in the response surface graphs (Fig. 4A). Regression models for both glucose yield variables were used for finding these optimal conditions. Numerical optimization methods provided several solutions for specified optimization conditions, from which only one was selected, based on the highest glucose yields and the lowest process harshness. The chosen optimal conditions were 198.28 °C and 16.00 min processing time. Hydrolysis and total glucose yields were predicted to reach 81.38% and 75.12% respectively. Predicted hydrolysis xylose yield was found to be 12.69% and predicted xylose filtrate yield was 9.34%.

3.4. *Hydrothermal post-treatment validation*

The accuracy of the optimized models for future sample predictions was then evaluated by validation. Additional raw biomass (i.e., prairie cordgrass) samples were subjected to clean fractionation and then were treated with hydrothermal

Table 4 – Results obtained from hydrothermal post-treatment (presented with ±1 standard deviation).

Exp.	Hydrolysis glucose yield [%]	Total glucose yield [%]	Xylose filtrate yield [%]	Hydrolysis xylose yield [%]	Acetic acid concentration in the hydrolyzate [g L^{-1}]	Acetic acid concentration in the filtrate [g L^{-1}]	HMF concentration in the hydrolyzate [g L^{-1}]	HMF concentration in the filtrate [g L^{-1}]	Furfural concentration in the hydrolyzate [g L^{-1}]	Furfural concentration in the filtrate [g L^{-1}]
1	58.13 ± 0.66	54.02 ± 0.20	0.00 ± 0.00	36.34 ± 2.02	0.37 ± 0.01	0.18 ± 0.02	0.00 ± 0.00	0.04 ± 0.00	0.01 ± 0.02	0.03 ± 0.00
2	72.95 ± 0.00	66.44 ± 0.00	1.37 ± 0.00	1.40 ± 0.00	0.27 ± 0.00	1.48 ± 0.00	0.11 ± 0.00	0.53 ± 0.00	0.32 ± 0.00	2.14 ± 0.00
3	62.26 ± 0.32	58.02 ± 0.26	0.47 ± 0.22	32.90 ± 2.05	0.38 ± 0.03	0.28 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.03 ± 0.03	0.07 ± 0.00
4	73.39 ± 0.49	67.57 ± 0.24	0.73 ± 0.00	1.13 ± 0.38	0.33 ± 0.04	1.61 ± 0.08	0.17 ± 0.02	0.70 ± 0.10	0.33 ± 0.01	2.27 ± 0.02
5	56.42 ± 2.03	52.23 ± 2.04	0.00 ± 0.00	33.62 ± 1.47	0.35 ± 0.01	0.21 ± 0.02	0.05 ± 0.00	0.02 ± 0.03	0.01 ± 0.01	0.03 ± 0.01
6	77.44 ± 0.48	70.63 ± 0.15	2.36 ± 1.15	1.46 ± 0.47	0.26 ± 0.00	1.60 ± 0.07	0.11 ± 0.00	0.52 ± 0.13	0.27 ± 0.01	2.21 ± 0.00
7	67.25 ± 2.50	62.26 ± 2.37	3.42 ± 1.49	24.04 ± 3.33	0.32 ± 0.02	0.47 ± 0.03	0.00 ± 0.00	0.07 ± 0.00	0.04 ± 0.01	0.21 ± 0.06
8	76.54 ± 2.95	70.77 ± 2.74	11.72 ± 0.92	16.49 ± 2.00	0.31 ± 0.01	0.98 ± 0.10	0.05 ± 0.00	0.11 ± 0.01	0.15 ± 0.03	0.92 ± 0.15
9	80.91 ± 0.00	75.10 ± 0.00	6.72 ± 1.15	23.27 ± 0.40	0.34 ± 0.00	0.72 ± 0.04	0.05 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	0.50 ± 0.10
10	80.92 ± 0.00	74.97 ± 0.00	9.85 ± 1.66	20.10 ± 0.90	0.32 ± 0.01	0.79 ± 0.09	0.03 ± 0.00	0.09 ± 0.02	0.11 ± 0.00	0.64 ± 0.08
11	81.28 ± 0.00	74.93 ± 0.00	9.94 ± 3.87	18.26 ± 2.40	0.28 ± 0.01	0.79 ± 0.12	0.05 ± 0.01	0.09 ± 0.02	0.07 ± 0.05	0.58 ± 0.20
12	74.93 ± 5.81	69.26 ± 5.31	10.98 ± 0.63	17.91 ± 0.49	0.31 ± 0.01	0.86 ± 0.06	0.06 ± 0.00	0.09 ± 0.00	0.09 ± 0.05	0.69 ± 0.10

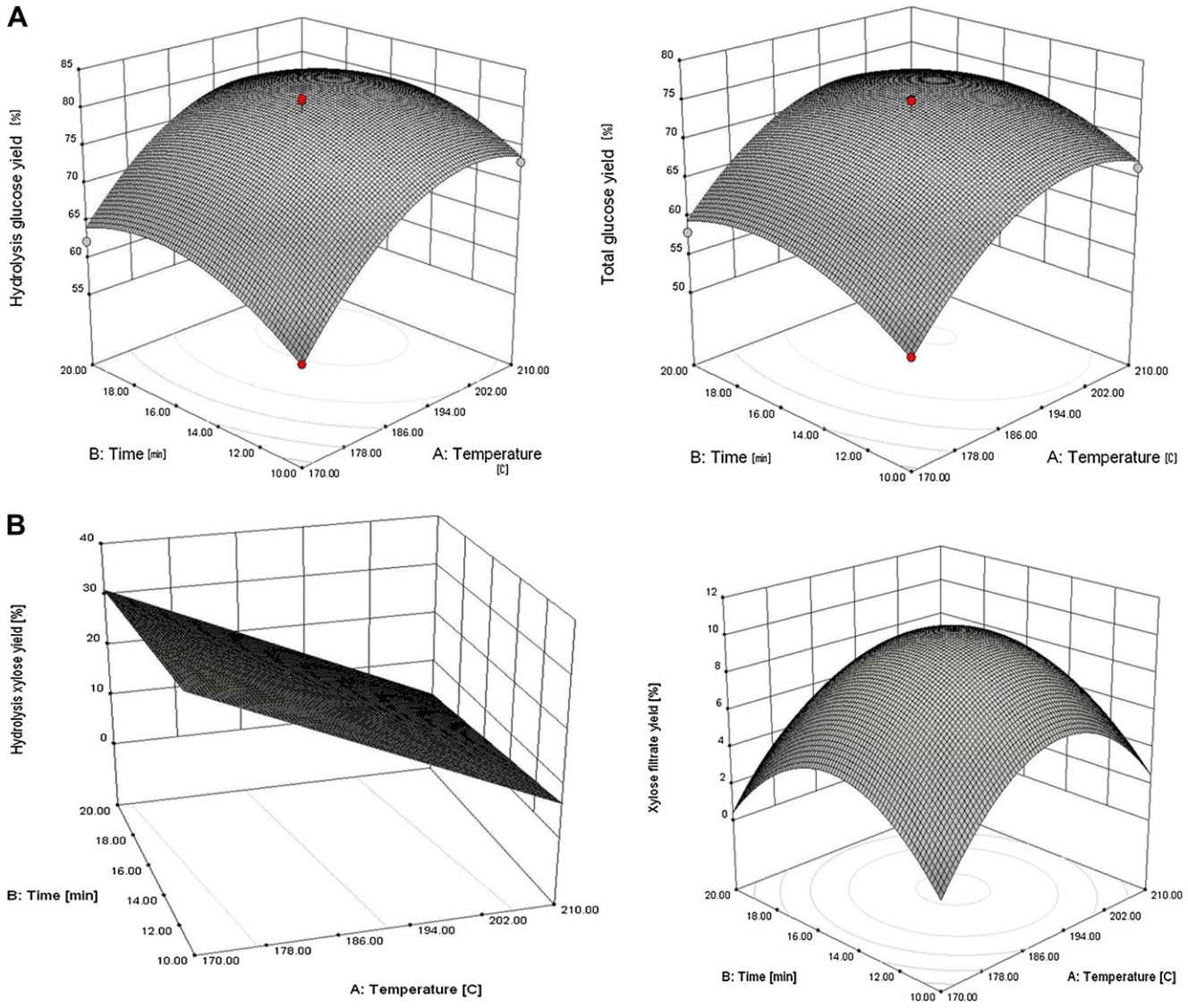

Fig. 4 – Response surface graphs for response variables [hydrolysis and total glucose yield, %; and hydrolysis and filtrate xylose yields, %] after hydrothermal post-treatment vs. time [min] and temperature [°C].

post-treatment at optimal conditions. Glucose yields were analyzed after the enzymatic hydrolysis, and xylose yields and by-product concentrations were also measured. The validation results for hydrolysis glucose yield (78.97 ± 3.98%) and total glucose yield (73.41 ± 3.71%) were both contained within the confidence intervals (77.86–84.90% and 71.90–78.32%, respectively), while hydrolysis xylose yield (15.48 ± 3.86%) and xylose filtrate yield (11.37 ± 1.10%) were both within prediction intervals (4.83–20.56% and 5.92–12.77%, respectively).

Enzymatic hydrolyzate contained low concentrations of acetic acid (0.36 ± 0.04 g L^{-1}), furfural (0.24 ± 0.03 g L^{-1}) and HMF (0.06 ± 0.01 g L^{-1}), all below suggested stress levels [35,37]. By-product concentrations measured in the filtrate were also relatively low, although concentrations of acetic acid (1.05 ± 0.14 g L^{-1}) and furfural (1.21 ± 0.26 g L^{-1}) exceeded yeast stress levels. HMF concentration in the filtrate was 0.12 ± 0.02 g L^{-1}, which was below yeast stress level. By-product concentrations were lower when compared to hydrothermal treatment applied to raw biomass [19].

3.5. *Simultaneous saccharification and fermentation (SSF)*

Fermentation ability of the solids obtained from the sequentially fractionated and hydrothermally treated prairie cordgrass was then tested using simultaneous saccharification and fermentation (SSF). Glucan content in the samples pretreated by the optimized clean fractionation and hydrothermal post-treatment was equal to 2.23 ± 0.02 g in 3.00 g of solids (suspended in 100 mL). This translated to 22.29 ± 0.17 g L^{-1} of glucan content in the fermentation mixture, which resulted in a theoretical ethanol concentration of 11.39 ± 0.08 g L^{-1}. The actual concentration of ethanol produced during SSF (after subtraction of the artificial concentration) was measured to be 10.61 ± 0.62 g L^{-1}. The relative ethanol yield was thus found to be equal to 93.18 ± 5.42%. Results of the monitored SSF process are presented in Figs. 5 and 6. Fig. 5

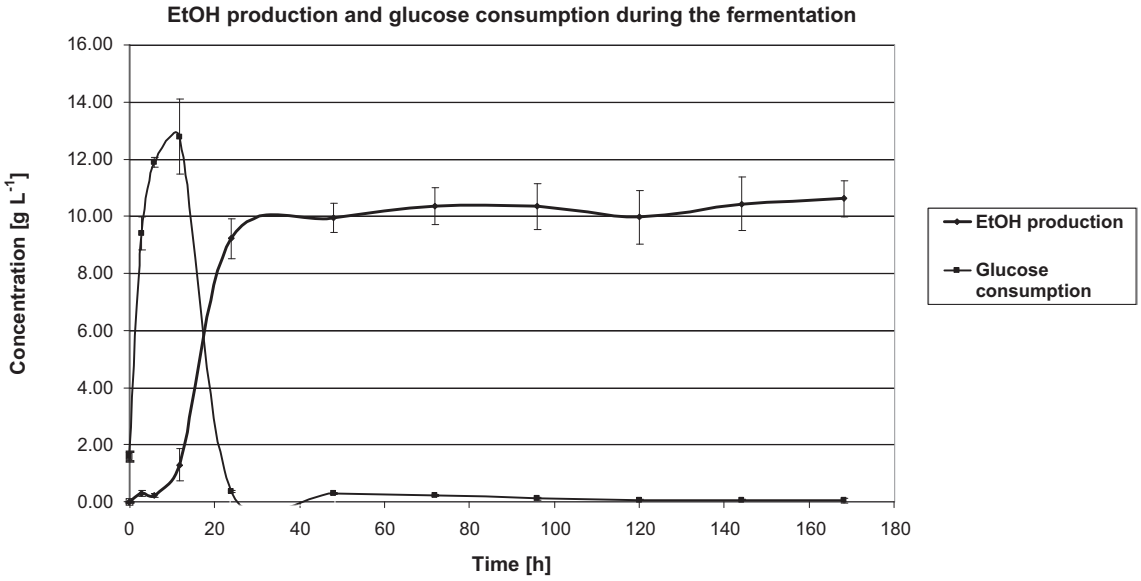

Fig. 5 – Ethanol production and glucose consumption during simultaneous saccharification and fermentation (SSF). Error bars represent ± one standard deviation.

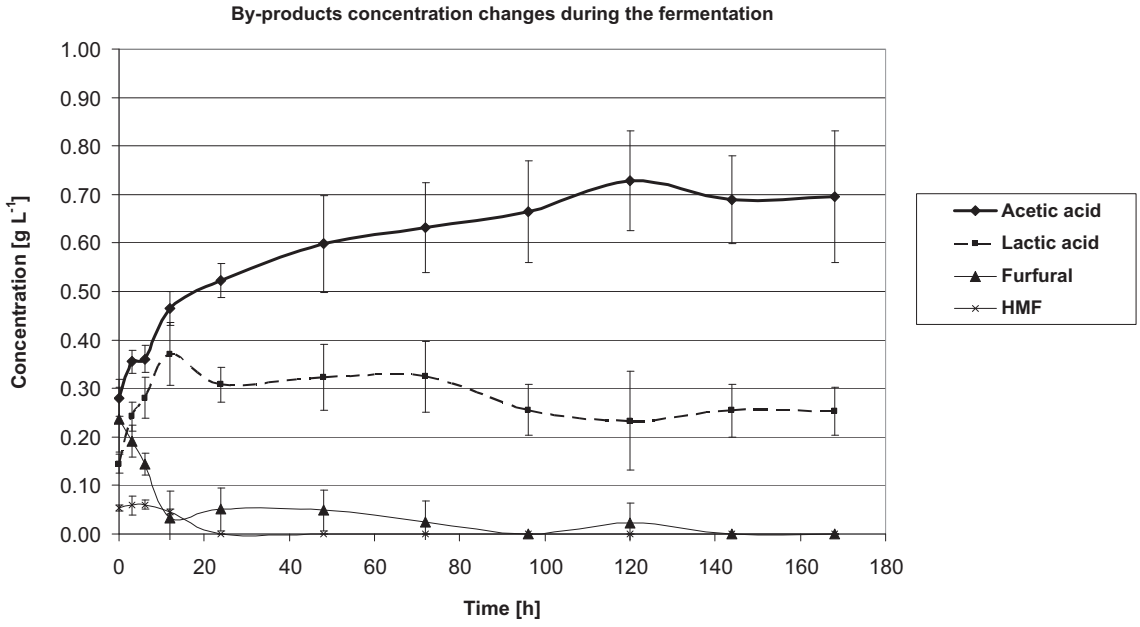

Fig. 6 – By-products concentrations during simultaneous saccharification and fermentation (SSF). Error bars represent ± one standard deviation.

shows ethanol production and glucose consumption over time, while Fig. 6 presents by-products (acetic acid, lactic acid, furfural and HMF) concentration changes over time. It can be observed that *S. cerevisiae* performance in ethanol production using pretreated prairie cordgrass was efficient and likely undisturbed. Ethanol yield corresponding to 95% glucose consumption occurred approximately just after 24 h, which suggests that the fermentation could be stopped after 24 h. By-product concentrations remained low from the beginning of the fermentation process. Furan concentrations decreased over time, which suggests that they were metabolized by the yeast [35].

4. Conclusion

Clean fractionation of prairie cordgrass alone produced solid fractions which resulted in low cellulose digestibility when subjected to enzymatic hydrolysis, thus a secondary treatment, hydrothermal post-treatment, was warranted. Optimization of catalyzed modified clean fractionation combined with hydrothermal post-treatment produced better results, valid regression models for lignin recovery and glucose yields (hydrolysis yield and total yield), and optimized conditions for each process.

A distinct optimum was found for the glucose yield models in the hydrothermal post-treatment, while a plateau was found for the lignin recovery model in the clean fractionation response surface graphs, which proves a correct choice of the experimental operability region. Validation of the optimization results showed that the prediction models were robust, and can be used to predict the behavior of future prairie cordgrass samples.

Application of optimal conditions for the combined processes resulted in relatively high lignin recovery and glucose yields, which leads to a conclusion that this pretreatment is effective. Xylose recovery from the process is possible; however, it would require combining different output streams, since xylose was divided amongst the solid and liquid fractions of both processes. The major part of xylose was released into the clean fractionation aqueous fraction, and could be recovered during solvent recycling.

Saccharification and fermentation (SSF) of the optimally pretreated samples resulted in high ethanol yield. No influence on the yeast performance of the inhibitory by-products released during enzymatic hydrolysis was observed. Thus, it appears that this integrated process produces enzymatically digestible and highly fermentable cellulose with good yields, along with extraction of organosolv lignin.

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Appendix A. Supplementary data

Supplementary data related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biombioe.2012.08.002.

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