Bioresource Technology 127 (2013) 92–99

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Catalyzed modified clean fractionation of switchgrass

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HIGHLIGHTS

- Catalyzed modified clean fractionation of switchgrass was evaluated and optimized.
- Catalyzed modified clean fractionation resulted in high lignin and glucose recoveries.
- Recovered lignin contained low ash and high Klason lignin content.
- Obtained cellulose was highly fermentable.

ARTICLE INFO

Article history:
Received 8 March 2012
Received in revised form 21 August 2012
Accepted 28 September 2012
Available online 6 October 2012

Keywords:
Clean fractionation
Organosolv treatment
Switchgrass
Ethyl acetate
Lignin

ABSTRACT

Switchgrass was used as a lignocellulosic feedstock for second generation ethanol production, after pretreatment using sulfuric acid-catalyzed modified clean fractionation based on NREL's (National Renewable Energy Laboratory) original procedure. Optimization of temperature, catalyst concentration and solvent composition was performed using Response Surface Methodology, and 59.03 ± 7.01% lignin recovery, 84.85 ± 1.34% glucose, and 44.11 ± 3.44% aqueous fraction xylose yields were obtained at 140.00 °C, 0.46% w/w catalyst concentration, 36.71% w/w ethyl acetate concentration, and 25.00% w/w ethanol concentration. The cellulose fraction did not inhibit the fermentation performance of *Saccharomyces cerevisiae* and resulted in an ethanol yield of 89.60 ± 2.1%.

1. Introduction

Switchgrass is considered one of the most attractive potential lignocellulosic ethanol feedstocks due to its ubiquitousness and low growing costs (Pimentel and Patzek, 2005; Sanderson et al., 2006). The energy input for switchgrass production is about half as much as that of corn; however, as with every lignocellulosic material, switchgrass requires a pretreatment step prior to ethanol production, which increases the cost of the final product by about 20% when dilute-acid hydrolysis is performed (Pimentel and Patzek, 2005). Therefore, finding a pretreatment method which requires lower energy input, or creates other possible revenue sources, could improve the economics of the production of switchgrass-based ethanol.

The concept of biorefinery is based on the principle of utilizing the full potential of lignocellulosic biomass, making ethanol production only one of the branches of a biorefinery. Other potential products of lignocellulosic origin include furfural, phenolics, xylitol, acetic acid, vanillin and many others and polymers such as phenolic resins, epoxy resins, polyurethane foams, nylon, and cellulose acetate (Kamm et al., 2006; Lora and Glasser, 2002; Pearl, 1967; Sarrouh et al., 2009).

In order to obtain value-added products in addition to ethanol, a pretreatment method must do more than just disrupt the lignocellulose structure to make it more susceptible to enzymatic digestion (Chandra et al., 2007). The first step that has to be applied in this concept is fractionation. One of the better known methods of fractionation, which has been practiced throughout the last century (in the pulp and paper industry) is organosolv treatment (Alvira et al., 2010; Taherzadeh and Karimi, 2008; Wyman, 1996).

There have been many alternatives of the organosolv process, each using different lignin solvents (e.g. alcohols, ketones, organic acids or esters) and processing conditions. The best known studies include projects like Lignol and ALCELL (developed in the 1970s) with application in the ethanol industry (Arato et al., 2005; Lora and Glasser, 2002; Pye and Lora, 1991). Lignin extracted in the organosolv process was found to be highly phenolic, and it had low molecular weight, which made it attractive for utilization in the production of chemicals (Arato et al., 2005). Another example

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http://dx.doi.org/10.1016/j.biortech.2012.09.131

of organosolv fractionation was developed in the 1990s by the National Renewable Energy Laboratory. "Clean fractionation", utilizes ketones (applied as lignin solvent) in a mixture with ethanol and water. In principle, clean fractionation produces three relatively clean fractions from biomass: solid cellulose pulp, an aqueous fraction rich in hemicellulose and an organic fraction rich in lignin. The aqueous fraction that contains hemicellulose sugars, furans and acetic acid, can be utilized in value-added products generation (Black et al., 1998; Chum et al., 1990). In general, different catalysts can be used in organosolv treatments to lower the processing temperature and increase lignin recovery and cellulose digestibility (Chum et al., 1990; Park et al., 2010). In the clean fractionation process sulfuric acid is used as catalyst (Black et al., 1998).

The present research focused on the optimization of a modified clean fractionation of switchgrass using ethyl acetate as a replacement for the methyl isobutyl ketone (MIBK) present in the original fractionation mixture. Ethyl acetate can be produced from biomass and has a lower health hazard and environmental impact as well as lower production cost than MIBK. The main objective was to maximize the amount of extracted lignin and the glucose yield produced by enzymatic hydrolysis. Additionally, a goal of this study was to lower the process severity by finding the lowest possible optimal temperature and catalyst concentration. Lignin recovery and fermentable glucose yields from enzymatic hydrolysis were the main response variables.

2. Methods

2.1. Switchgrass

Switchgrass harvested near Brookings (South Dakota) was ground to pass a 1 mm sieve (Thomas-Wiley Laboratory Mill, Model 3375-E15, Thomas Scientific, USA). The composition of the raw material was analyzed by acid hydrolysis (Sluiter et al., 2008b). The switchgrass contained 36.69 ± 0.94% glucose, 20.72 ± 0.68% xylose, 2.94 ± 0.18% arabinose, 1.30 ± 0.01% galactose, 0.20 ± 0.05% mannose, 20.45 ± 0.41% lignin and 2.61 ± 0.30% ash, 4.47 ± 0.11% water soluble extractives and 3.17 ± 0.11% ethanol soluble extractives and 6.47 ± 1.00% moisture.

2.2. Experimental design

The experimental design, data analysis and optimization were based on Response Surface Methodology, with application of statistical software (Design Expert version 8.0.1.0). Four factors were included in the optimization procedure: temperature, catalyst concentration, and ethyl acetate and ethanol concentration in the solvent mixture. The experiment was designed using Small Central Composite Design (SCCD) with an α value of 1.681, chosen to ensure rotatability of the design (Table 1). An α value (specified by a coded value) represents the distance of the star points from the center point for all factors. Star points represent runs at which all but one factor were set at their mid-levels. The experimental region included 8 factorial design points, 8 axial points, and 4 replicates of center point. Two replications were performed for all factorial and axial design points. Since the goal of this study was to lower the severity of the process, the high factorial point (+1.00) for temperature was chosen based on the original clean fractionation process developed by the NREL (140 °C), as well as the high star point (+1.68) of the catalyst (0.57% w/w) (Black et al., 1998). Low factorial points (−1.00) for these factors were chosen to ensure that all solvent components would be vaporized at the low star point (−1.68) and to ensure a positive value of the catalyst concentration at the low star point. Factorial points for the ethyl acetate and ethanol concentration in the solvent mixture were selected based on the formation of a one-phase mixture with water. The phase diagram presented by Resa et al. (2006) was used for this selection.

Response variables for optimization included lignin recovery, hydrolysis glucose yield and total glucose yield. Optimization of clean fractionation was performed using lignin recovery as the primary response variable and glucose yields as secondary variables. Xylose yields were not included in the optimization, but their values for the optimal conditions were predicted using the developed models.

All the tests were performed in duplicates.

Statistical model validity was tested by calculating R-squared values: R-squared raw, adjusted, and predicted. R-squared adjusted corrects the R-squared raw for the non-significant terms present in the model and sample size, avoiding model overfitting. R-squared predicted evaluates the ability of the model to fit future samples (Khuri and Cornell, 1996).

2.3. Catalyzed modified clean fractionation

The clean fractionation procedure was based on NREL's batch process (Black et al., 1998) using an alternative solvent composed of ethyl acetate, ethanol and water. Ethyl acetate replaced methyl isobutyl ketone (MIBK), in order to lower toxicity (NFPA Health Hazard of ethyl acetate is at level 1, while for MIBK is at level 2) and the cost of the solvent as industrial MIBK is about twice the price of ethyl acetate (Li, 2012). Ethyl acetate also shows higher affinity to lignin as a solvent, based on the Hildebrand's constant (δ) (Davison, 1982). The process was catalyzed by sulfuric acid in order to initiate carbohydrate-lignin bonds cleavage at lower processing temperatures (Black et al., 1998).

Digestion was performed in a custom-made experiment setup consisting of an aluminum heating block and six stainless steel reactor tubes, each with 250 mL capacity and sealed tightly with Teflon gaskets. Temperature was controlled by auto-tune temperature controllers (CN9121A, Omega) integrated with thermocouples connected to the heating block. The temperature profile in each reactor was monitored with thermocouples, installed on top of reactor screw caps, by Lab View 8.2 software utilizing a data acquisition device (OMB-DAQ-56, Omega), while pressure was monitored by gauges installed on the reactor caps.

Biomass loading was equal to 10% w/w of dry weight, and the entire reaction mixture (with the solvent) weighed 100 g. Heating time ranged between 20 and 40 min, while cooling time was around 20 min. Reaction time was 20 min for all experiments. Based on previous studies, time was found to be a non-significant factor in the fractionation process up to 1 h.

After the reaction, the solids were separated from the liquid by vacuum filtration and liquid permeate was collected. The solids were washed with 20–50 mL of ethyl acetate to avoid reprecipitation of lignin on the cellulose fibers, and then washed with 80–100 mL of deionized water. Subsequently, the solids were flushed with about 1 L of water, in order to remove the remaining solvent. The liquid permeate was combined with the ethyl acetate/deionized water mixture used in the first solid wash. The ethyl acetate/deionized water mixture disrupted the liquid–liquid equilibrium and induced phase separation. The aqueous fraction was separated from the organic fraction using a separatory funnel. The organic fraction, containing mainly ethyl acetate, was evaporated to obtain a solid lignin-rich fraction. The aqueous fraction, which contained xylose and traces of glucose, by-products and remaining solvent, was analyzed by High Performance Liquid Chromatography (Agilent Technologies System 1200) with Biorad Aminex 87H Column using NREL's standard protocol (Sluiter et al., 2008a). The solid fraction was subjected to enzymatic hydro-

Table 1
Experimental design for clean fractionation optimization.

Experiment no.	Temperature (°C)	Coded value	Sulfuric acid concentration (%w/w)	Coded value	Ethyl acetate concentration (%w/w)	Coded value	Ethanol concentration (%w/w)	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

lysis to evaluate its digestibility (Selig et al., 2008). The purity of the fractions of lignin and cellulose were evaluated by Klason lignin measurements and enzymatic hydrolysis, respectively.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis was used to evaluate the solid fraction's digestibility and therefore its glucose yield. Enzymes used during the hydrolysis included cellulase (Novozymes, NS50013) and β-glucosidase (Novozymes, NS50010) in doses of 15 FPU/g DM and 60 CBU/g DM. The enzymatic hydrolysis procedure followed NREL's standard protocol (Selig et al., 2008). Biomass loading was equal to 3% dry weight, and the entire volume of the hydrolysis mixture was 10 mL. The pH was adjusted to 4.8 as recommended for best enzyme performance, and the process was run for 72 h. The process was performed in scintillation vials inside a shaking bed incubator (Incubated Tabletop Orbital Shaker Model 420, Fisher Thermo Scientific) set at 50 °C and 150 rpm. The enzymatic hydrolyzates were analyzed for glucose, xylose and by-products concentrations on an HPLC instrument (Agilent Technologies System 1200 with a Biorad Aminex 87H column) (Sluiter et al., 2008a).

2.5. Simultaneous saccharification and fermentation (SSF)

High glucose yields in the enzymatic hydrolysis would suggest a high amount of substrate for ethanol production; however, since performance of microorganisms can differ based on the feedstock, a simultaneous saccharification and fermentation (SSF) process was conducted to test the yeast's (*Saccharomyces cerevisiae* D_5A (No. 200062), obtained from ATCC (Manassas, VA, USA)) ability to digest the pretreated samples. The procedure followed NREL's standard protocol (Dowe and McMillan, 2008).

The yeast culture was grown on a solid medium (sterile agar, peptone and glucose). The inoculum was prepared by transferring one of the yeast cultures into a sterile growth medium (5% glucose, 0.5% yeast extract in deionized water) and incubating at 35 °C, 200–250 rpm for 24 h prior to fermentation start-up.

The start-up mixture (total volume of 100 mL) was prepared in 250 mL Erlenmeyer flasks with yeast locks, following the enzymatic hydrolysis pattern (the same enzyme doses and biomass loading) in order to be able to compare the results between both processes. Yeast extract was added as a nutrients source (0.5 g/100 mL) and tetracycline was used to inhibit bacterial growth (0.3/100 mL). *S. cerevisiae* culture was added at 1/100 mL, just before the start of the process.

The mixture was incubated at 35 °C for 168 h, with constant mixing at 90 rpm. Sampling was conducted aseptically after 0, 3, 6, 12, 24, 48, 72, 96, 120, 144 and 168 h. Subsequently, the samples were analyzed for concentrations of ethanol, residual glucose, hemicellulose sugars and generated by-products by HPLC (Agilent Technologies System 1200 with Biorad Aminex 87H column).

2.6. Glucose yields

Two values for glucose yield (%) were calculated: hydrolysis glucose yield (expressing cellulose digestibility) and total glucose yield (expressing the entire pretreatment efficiency, by accounting for glucose losses into the aqueous fraction). In each calculation the final glucose concentration in the hydrolyzates, measured by HPLC, was divided by the glucose content either in the raw material (total glucose yield) or in the pretreated material (hydrolysis glucose yield). Both glucose yields are expressed in the following equations (Eq. (1) and (2)):

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

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

2.7. Lignin recovery

The weight of the lignin-rich solid fraction obtained after organic fraction evaporation was used to calculate initial lignin recovery (used for optimization) by comparing it to the structural lignin content in the raw material. Lignin from the optimally pretreated samples was analyzed for acid-insoluble lignin (Klason lignin) and ash content, in order to evaluate its actual purity. Lignin recovery (%) calculation is presented by Eq. (3):

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

2.8. Xylose yields

Xylose yield (%) was calculated for both the enzymatic hydrolyzate and the aqueous fraction from clean fractionation in order to monitor its distribution between solid and liquid fractions. The yields were calculated by comparing of the concentrations measured in the aqueous fractions with those in the raw material. The calculations are presented by Eq. (4) and (5):

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

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

2.9. Ethanol yield

The microorganisms' performance was examined mainly by relative ethanol yield (%), which was calculated using the enzymatic hydrolysis results (eliminating the enzymes' efficiency aspect) and glucose-to-ethanol conversion stoichiometry (Eq. (6)). Theoretical ethanol yield was calculated using Eq. (7), and the result was compared with the actual ethanol yield measured at the end of the SSF process (Eq. (8)). To obtain true final ethanol yield, the actual ethanol yield was corrected for the tetracycline peak, as the antibiotic co-eluted with the ethanol.

3. Results and discussion

3.1. Clean fractionation optimization

The highest lignin recovery (72.71 ± 2.08%) was obtained at the high factorial points of temperature (140 °C), relatively high catalyst concentration (0.46% w/w) and ethyl acetate concentration (50% w/w) and at the low factorial point of ethanol concentration (10% w/w) in the solvent mixture (experiment 1, Table 2). This result is comparable with that obtained by Brudecki et al. (2012), who used prairie cordgrass as feedstock and applied the original clean fractionation process using MIBK as the lignin solvent.

The highest hydrolysis glucose yield (96.33 ± 0.10%) and the highest total glucose yield (92.32 ± 0.05%) were found in the sample treated at the high star point of temperature (150.23 °C) and center points of all the other factors (catalyst concentration at 0.31% w/w, ethyl acetate concentration at 32.50% w/w and ethanol concentration at 22.50% w/w), which is presented in Table 2 (experiment 10).

Xylose yields were calculated for both the enzymatic hydrolyzate and the clean fractionation aqueous fraction (Table 2). The highest xylose yield was observed in the aqueous fraction (67.92 ± 2.68%) of experiment 2, which represented high factorial points for temperature (140 °C) and catalyst concentration (0.46% w/w) and low factorial points for ethyl acetate (15% w/w) and ethanol (10% w/w) concentration in the solvent mixture. Hydrolysis xylose yield did not exceed 33.46 ± 0.30% (experiment 7). Acetic acid was the only by-product detected in the aqueous and solid fraction hydrolyzates. The aqueous fractions yielded high acetic acid concentrations, in some experiments exceeding 50 g/L

$$C_6H_12O_6 \rightarrow 2C_2H_5OH + CO_2 \quad (6)$$

$$\text{Theoretical ethanol conc.}[g/L] = \text{Glucose conc. after enzymatic hydrolysis } [g/L] \times 0.51 \quad (7)$$

$$\text{Relative ethanol yield}[\%] = \frac{\text{Final actual ethanol concentration}[g/L] - \text{Initial artificial ethanol concentration}[g/L]}{\text{Theoretical ethanol concentration}[g/L]} \times 100\% \quad (8)$$

Table 2
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)	Acetic acid concentration in the aqueous fraction (g/L)
1	72.71 ± 2.08	73.38 ± 3.00	70.61 ± 2.80	49.45 ± 1.51	15.49 ± 0.55	0.21 ± 0.00	75.92 ± 5.83
2	15.22 ± 1.41	73.85 ± 0.51	70.99 ± 0.49	67.92 ± 2.68	16.16 ± 0.58	0.18 ± 0.03	43.90 ± 5.33
3	37.37 ± 0.70	33.60 ± 1.41	33.26 ± 1.37	5.22 ± 0.63	28.41 ± 1.24	0.27 ± 0.02	12.65 ± 1.00
4	17.70 ± 0.00	28.88 ± 2.79	28.70 ± 2.74	2.09 ± 1.39	18.31 ± 1.99	0.13 ± 0.02	22.42 ± 0.34
5	14.73 ± 4.88	35.37 ± 3.18	35.01 ± 3.18	3.45 ± 0.43	22.49 ± 0.40	0.22 ± 0.01	9.54 ± 0.11
6	18.16 ± 0.00	20.08 ± 2.35	19.99 ± 2.33	0.93 ± 1.32	9.34 ± 3.24	0.07 ± 0.02	9.98 ± 2.61
7	16.46 ± 2.43	41.49 ± 0.50	40.93 ± 0.45	8.06 ± 0.88	33.46 ± 0.30	0.28 ± 0.02	34.62 ± 0.08
8	1.96 ± 0.00	16.58 ± 0.63	16.51 ± 0.63	0.00 ± 0.00	5.86 ± 0.16	0.06 ± 0.02	11.08 ± 1.01
9	11.31 ± 0.70	19.18 ± 0.86	19.10 ± 0.85	0.29 ± 0.41	9.51 ± 1.09	0.12 ± 0.03	30.65 ± 1.94
10	58.74 ± 0.31	96.33 ± 0.10	92.32 ± 0.05	55.23 ± 0.18	21.25 ± 1.04	0.25 ± 0.00	51.05 ± 0.80
11	10.80 ± 0.70	12.42 ± 0.59	12.37 ± 0.59	0.00 ± 0.00	3.18 ± 0.56	0.00 ± 0.00	0.94 ± 0.52
12	62.34 ± 2.08	66.21 ± 0.36	64.81 ± 0.45	31.24 ± 2.23	31.08 ± 0.69	0.31 ± 0.02	62.16 ± 5.08
13	6.63 ± 1.75	32.38 ± 1.17	32.12 ± 1.15	3.93 ± 0.81	17.54 ± 0.56	0.15 ± 0.00	10.56 ± 0.17
14	63.84 ± 3.43	46.78 ± 2.18	45.64 ± 1.89	22.92 ± 4.70	27.17 ± 3.48	0.28 ± 0.01	51.18 ± 1.57
15	15.48 ± 3.82	35.42 ± 0.82	35.08 ± 0.81	9.12 ± 1.65	18.12 ± 0.56	0.21 ± 0.00	44.22 ± 4.29
16	14.50 ± 1.05	36.17 ± 1.62	35.78 ± 1.56	4.98 ± 0.78	31.10 ± 1.28	0.28 ± 0.03	21.27 ± 0.32
17	49.13 ± 0.00	45.22 ± 1.50	44.73 ± 1.36	9.67 ± 3.66	28.44 ± 0.37	0.29 ± 0.01	47.86 ± 3.36
18	40.78 ± 0.00	47.39 ± 1.70	46.78 ± 1.72	12.72 ± 1.00	28.46 ± 1.84	0.33 ± 0.05	51.95 ± 3.22
19	39.72 ± 0.00	43.26 ± 0.10	42.71 ± 0.10	11.56 ± 0.20	28.07 ± 0.97	0.31 ± 0.01	54.26 ± 0.96
20	45.96 ± 3.85	44.78 ± 1.39	44.33 ± 1.40	8.80 ± 0.16	29.78 ± 0.76	0.32 ± 0.02	49.14 ± 0.84

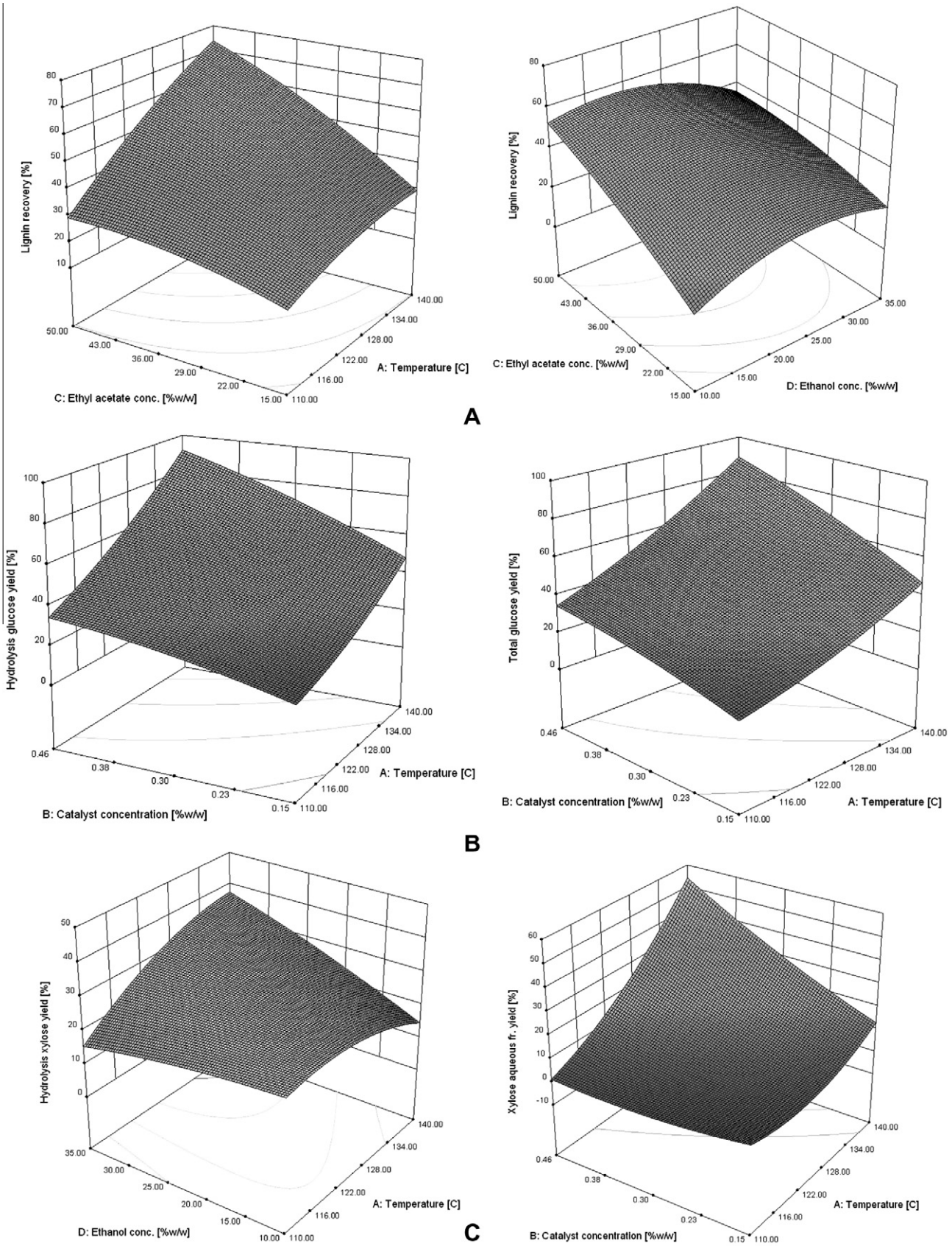

Fig. 1. Response surface graphs for response variables (lignin, %; hydrolysis and total glucose yields, %; and hydrolysis and aqueous fraction xylose yields, %) vs. significant factor interactions (catalyst concentration, %w/w; ethyl acetate concentration, %w/w; ethanol concentration, %w/w; temperature, °C) after clean fractionation process.

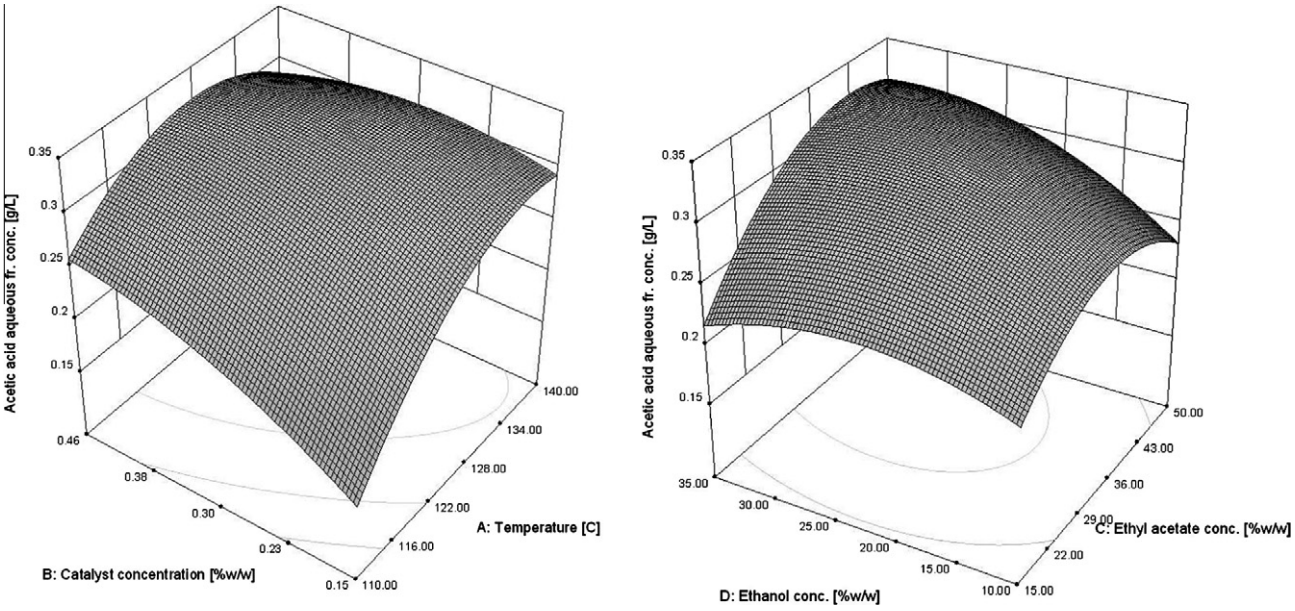

Fig. 2. Response surface graphs for acetic acid concentration in the aqueous fraction (g/L) vs. the significant factor interactions (catalyst concentration, %w/w, temperature, °C, ethanol concentration, %w/w and ethyl acetate concentration, %w/w) after clean fractionation.

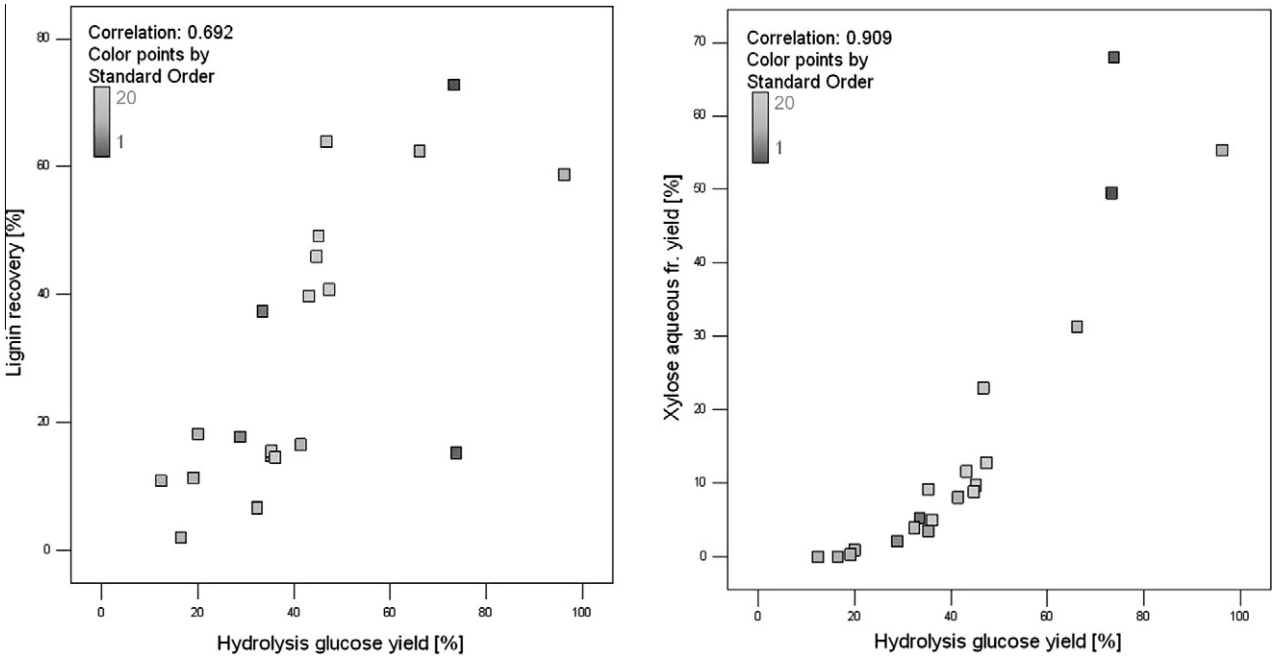

Fig. 3. Correlation between hydrolysis glucose yield and lignin recovery/xylose aqueous fraction yield.

(Table 2). The main source of acetic acid was the hydrolysis of ethyl acetate triggered by acidic conditions (Patai, 1969), not the deacetylation of hemicellulose. Acetic acid concentration in the enzymatic hydrolyzate did not exceed 0.5 g/L, which is the suggested lower threshold of its inhibitory influence on microorganisms'. No furfural or hydroxymethyl furfural (HMF) were detected in either fraction.

Statistical models were developed for the response variables, which were used for numerical optimization (lignin recovery and glucose yields), xylose yields, and acetic acid concentration in the aqueous fraction (to model ethyl acetate hydrolysis). No transformations of data were necessary based on the Box–Cox plot. Analysis of variance showed that all factors were significant (p-value < 0.05) for lignin recovery except ethanol concentration in the solvent mixture. Significant interactions for the lignin model included temperature interaction with ethyl acetate and ethanol concentration (p-value < 0.05) in the solvent mixture (Fig. 1A). Ethanol concentration as a model term was non-significant for the glucose yield models (p-value > 0.05), while all three remaining factors were found significant (p-value < 0.05). Significant interactions in hydrolysis glucose and total glucose yield models included the temperature interaction with catalyst concentration (Fig. 1B) as well as the catalyst concentration interaction with ethanol concentration (not presented). Xylose yield in the aqueous fraction was

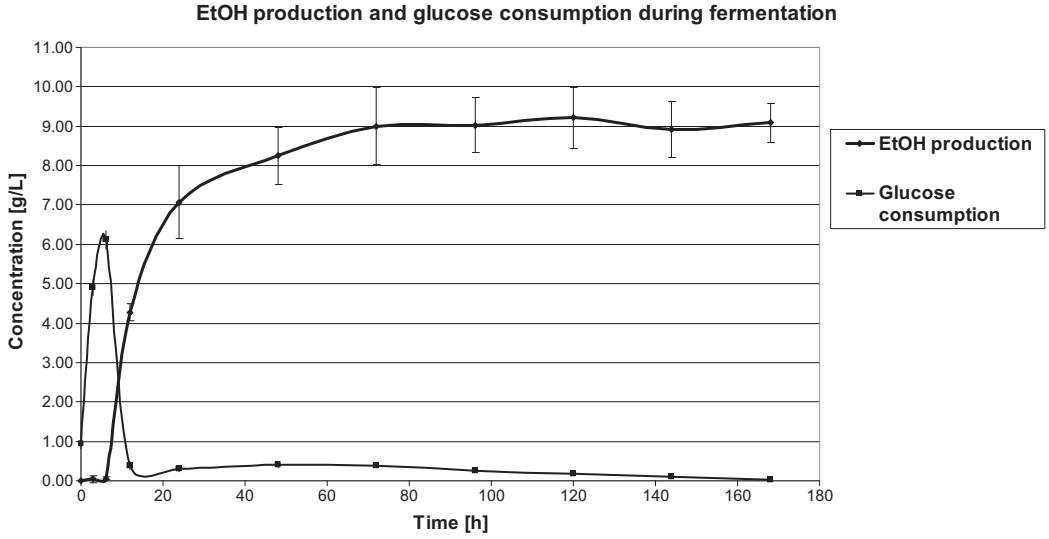

Fig. 4. Ethanol production and glucose consumption during SSF.

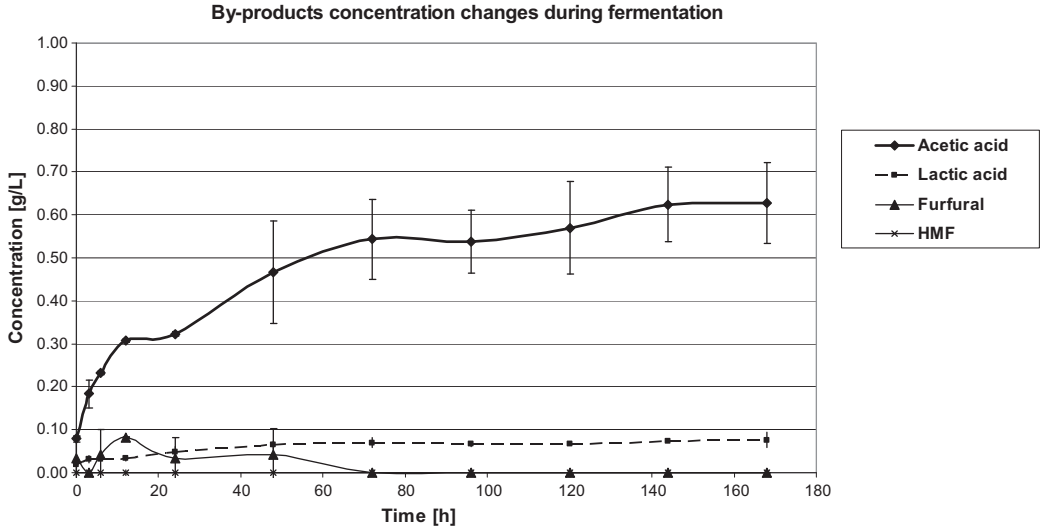

Fig. 5. By-products concentration changes during SSF.

mostly influenced by temperature and catalyst concentration and their interaction (Fig. 1C). All factors were significant in the hydrolysis xylose yield model, and the only significant interaction was between temperature and ethanol concentration (Fig. 1C). Acetic acid release into the aqueous fraction was dependent upon all of the factors, and most of their interactions. The temperature's interaction with catalyst concentration as well as the interaction of ethyl acetate with ethanol influenced the release of acetic acid the most (Fig. 2).

All R-squared coefficients for the models were close to 1.00, which suggests that the models are well fitted and can be used for future samples.

Cellulose digestibility (hydrolysis glucose yield) increased with increasing delignification (lignin recovery) and increasing xylose removal into the aqueous fraction (xylose aqueous fraction yield), which suggests a positive correlation (Fig. 3). This finding supports the hypothesis that cellulose fibers become more susceptible to enzyme digestion once the carbohydrate-lignin bonds are cleaved and lignin as well as hemicellulose is removed from the lignocellulosic structure (Chandra et al., 2007).

Numerical methods provided several solutions for specified optimization conditions (based on maximizing three response variables, and suggested different values of the four optimized factors), but only the one with the highest desirability (0.987) was chosen. Optimal conditions were found at 140.00 °C, 0.46% w/w catalyst concentration, 36.71% w/w ethyl acetate concentration, and 25.00% w/w ethanol concentration, which resulted in a predicted lignin recovery of 74.02%, and a 94.29% and 91.02% hydrolysis and total glucose yields, respectively. Hydrolysis xylose yield was predicted at 32.83% and xylose aqueous fraction yield at 51.60%.

3.2. Clean fractionation validation

Optimization results were tested in order to validate the accuracy of the models. Lignin recovery was 59.03 ± 7.01%, glucose yields were 84.85 ± 1.34% and 82.21 ± 1.31% for hydrolysis and total glucose yields, respectively, xylose yields were 27.14 ± 1.93% and 44.11 ± 3.44% for hydrolysis xylose yield and xylose aqueous fraction yield, respectively. The only by-product detected was

acetic acid, reaching 48.18 ± 1.99 g/L for the aqueous fraction but remaining low in the enzymatic hydrolyzate (0.29 ± 0.03 g/L). No formation of furans was observed. Measured values of all response variables were confined within their prediction intervals (57.63–90.42% for lignin recovery, 84.62–100.00% for hydrolysis glucose yield, 82.08–99.96% for total glucose yield and 42.37–60.83% for xylose aqueous fraction yield), except for xylose hydrolysis yield, which still was within the tolerance interval (22.77–42.89%). These results show that the models accurately predicted the responses, and that the optimization can be considered valid.

The Klason lignin and ash content in the extracted lignin were equal to 73.49 ± 1.23%, and 0.44 ± 0.01%, respectively.

3.3. Simultaneous saccharification and fermentation (SSF)

The results of enzymatic hydrolysis were a base for calculating theoretical ethanol yield, which could be achieved during fermentation if the microorganisms' performance was at its maximum. The glucose concentration during enzymatic hydrolysis was 19.84 ± 0.61 g/L and resulted in a theoretical ethanol concentration of 10.14 ± 0.31 g/L. The actual ethanol concentration measured at the end of SSF (after correction for tetracycline) was equal to 9.09 ± 0.5 g/L, which was 89.60 ± 2.1% of theoretical yield. Ethanol production along with glucose consumption during monitored SSF is shown in Fig. 4, from which it can be observed that the fermentation rate dropped significantly after ~24 h and maximum ethanol yield was achieved after ~70 h. By-product formation during SSF is presented in Fig. 5. Only acetic acid was produced in amounts higher than the potential inhibitory threshold, and furan concentrations decreased over time. It has been shown (Klinke et al., 2004) that furans present in the fermentation broth can boost ethanol yield. High ethanol yield suggests that the acetic acid concentration did not influence the yeast's performance. Lactic acid concentrations detected were too low to suggest bacterial contamination.

4. Conclusions

Optimization of the catalyzed modified clean fractionation process was performed for switchgrass. Statistical models developed in the optimization process were confirmed to be accurate, and can be used for future samples.

The results revealed that clean fractionation with ethyl acetate as is an effective pretreatment method for switchgrass. The process produces highly fermentable cellulose (89.60 ± 2.1% ethanol yield) and pure lignin (73.49 ± 1.23% Klason lignin). These findings prove that clean fractionation with ethyl acetate has the potential for implementing the biorefinery concept and utilizing biomass for production of a wide range of value-added products in addition to biofuels.

Acknowledgements

This work was supported by funding from the South Dakota Center for Bioprocessing Research and Development, Sun Grant Initiative and SDSU Agricultural Experiment Station.

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