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Optimization of clean fractionation processing as a pre-treatment technology for prairie cordgrass

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ABSTRACT

The main objective of this study was to fractionate prairie cordgrass (PCG) obtaining the highest cellulose digestibility. Following clean fractionation (CF) processing, the PCG lignocellulosic biomass was fractionated into three main building blocks: cellulose, hemicellulose and lignin. Effects of processing factors such as time, temperature, catalyst concentration and organic solvent mixture composition were evaluated. Organic solvent–aqueous mixture contained methyl isobutyl ketone (MIBK), ethanol and water in different proportions. Sulfuric acid was used as a catalyst. In order to evaluate the degree of pre-treatment, enzymatic saccharification was employed on the cellulose fraction obtained from the CF process. Response surface methodology was used for process optimization and statistical analysis. Optimal conditions (39 min, 154 °C, 0.69% catalyst and 9% MIBK) resulted in 84% glucose yield and 87% acid insoluble lignin (AIL).

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1. Introduction

Increasing energy demands, depletion of fossil fuels as well as environmental and sustainability aspects have huge influences on interest and development of renewable energy systems. Different renewable sources of energy such as wind, solar, geothermal and biomass are alternatives to fossil fuels, but bioethanol is still one of the most attractive options (Tan et al., 2008). Development of the alcohol industry can be observed by the increasing number of ethanol plants. Currently there are more than 200 operating ethanol plants in the USA with production exceeding 14 MMGY (RFA, 2010). EU input has been relatively small, with only 10% worldwide; however, biofuels targets of 5.75% of renewable fuels in transport petrol and diesel by 2010, and 10% by 2020 have been implemented (European Commission, 2011).

Conversion of biomass to ethanol depends on many aspects; however type of feedstock, pre-treatment and enzyme loading is crucial for the process. Since any easily convertible source of sugar or starch can be utilized as bioethanol feedstock, corn and sugarcane are the most commonly exploited conventional crops. Ethanol production technologies from sugar and starch feedstocks are still developing. Some of the recent key technical and economic challenges include utilization of new microorganisms capable of fermenting C5 sugars, kinetics models, fermentation inhibition aspects and increasing productivity by better utilization of yeast or higher loading of substrate (e.g. very high gravity (VHG) fermentation) (Bai et al., 2008). However current increases of food prices caused by increased energy prices have forced producers to search for other types of biofuels feedstock.

Second generation biofuels (e.g. lignocellulosic bioethanol), based on lignocellulosic biomass, are becoming a viable alternative. Low-cost agricultural residues such as corn stover, sugar cane bagasse and wheat straw are potential materials for lignocellulosic ethanol production (Sanchez and Cardona, 2008). Also, perennial warm-season grasses such as prairie cordgrass (PCG), switchgrass (SG) and big bluestem (BBS) are alternative feedstocks. Apart from being abundant and non-edible, their advantage is higher content of cellulose compared to crop residues and cool-season grasses (Lee et al., 2007). PCG grows throughout the Northeast, Great Lakes, Midwest states and Canada. PCG is one of the dominant grasses of the tall grass prairie (up to 2.5 m tall), growing mainly in low and wet areas; also it grows faster than other tall grass prairie grasses. An advantage of PCG over SG and BBS is better adaptation to soils that are too wet and not sufficiently aerated. Coarseness of PCG limits its use as animal feed. Large annual yield (production up to 9696 kg DM (dry matter) $ha^{-1}$of prairie cordgrass from eastern South Dakota) (Boe and Lee, 2007), higher bulk density (170–197 kg/m^3) (Karunanithy and Muthukumarappan, 2010) as well as relatively high cellulose content makes PCG an attractive feedstock for ethanol production (Cybulska et al., 2009).

The complex structures of lignocellulosic biomass and their recalcitrance to enzyme action necessitate pre-treatment as a

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crucial step for lignocellulosic bioethanol production. The main goal of pre-treatment is to alter the structure of lignocellulosic material to increase enzyme accessibility, thus improving the digestibility of the cellulose (Mosier et al., 2005). Pre-treatment technologies can be classified into biological, physical, chemical and physicochemical. However, recent research trends show that physicochemical pre-treatment could be most favorable for biofuels production. Hydrothermal, steam explosion, dilute acid, ammonia fiber explosion (AFEX) and lime treatment are the leading technologies for biomass pre-treatment (Wyman et al., 2005).

Clean fractionation (CF) is a type of organosolv pre-treatment; its purpose is to separate lignocellulosic material into cellulose, hemicellulose and lignin. CF is suitable for a wide range of biomass feedstocks, including wood and herbaceous biomass. A single-phase mixture composed of water, immiscible organic solvent (e.g. methyl isobutyl ketone (MIBK)) and ethanol in different proportions, can be used as digesting liquor. The process operates at reasonable temperatures (100–220 °C) and pressures. Catalyst is added to reduce reaction temperature to about 140 °C. After digestion, the amount of water or organic solvent is adjusted to induce phase separation. The organic fraction will be rich in lignin, while hemicellulose components will be in the aqueous fraction, and cellulose will be present in a solid phase. The two liquid phases have very little cross-contaminant of components from one phase into other. Lignin is obtained by evaporation of the organic solvent. Successful runs with efficient fractionation were performed on poplar and aspen wood chips in batch and percolation reactors. Around 50% of pulp, 20% of lignin and 30% of hemicellulose were obtained (Black et al., 1998).

The lack of commercial lignocellulosic-based biorefineries is a consequence of poor economics when ethanol is the sole source of the revenue stream. For instance, in the Lignol Biorefinery process, ethanol revenue represents only one-third of total revenues of the facility, while lignin- and hemicellulose-based products generate the remaining revenues (Arato et al., 2005). Therefore utilization of the three main fractions of lignocellulosic materials (cellulose, hemicellulose and lignin) is very important for commercialization of biorefineries.

The objective of this study was to optimize processing parameters of PCG using clean fractionation, including time, temperature, catalyst concentration and organic solvent mixture composition. The fractionation process was evaluated based on the highest glucose yield, which was the main goal in this study, while the secondary priority was maximizing amount of extracted lignin and hemicellulose.

2. Methods

2.1. Sample preparation and chemicals

PCG biomass was harvested from a local farm near Brookings, SD. PCG was air dried and ground in a hammer mill (Speedy King, WinonaAttrition Mill Co., MN, USA) using an8 mm sieve for size reduction. In order to have uniform material for the CF experiment, PCG was subsequently ground to pass through a 1-mm screen (Thomas-Wiley Laboratory Mill, Model 3375-E15, Thomas Scientific, USA). The size of the grass was chosen based on the fact that the size of 1 mm gives fairly uniform material, which is important in such small quantities used in the trials. The moisture content of the ground PCG samples was determined as described in NREL/TP-510-42621 (Sluiter et al., 2008a). Compositional analysis of ground samples was performed by acid hydrolysis according to NREL procedure NREL/TP-510-42618 (Sluiter et al., 2008c). Biomass composition was analyzed to be: 37.43 ± 0.59% glucose, 15.48 ± 0.25% xylose, 2.83 ± 0.13% arabinose, 1.40 ± 0.32% galactose, 0.50 ± 0.11% mannose, 16.40 ± 0.67% acid insoluble lignin and 4.84 ± 0.08% ash, calculated per dry matter (DM). The chemicals used in the analyses of the hydrolysates by high performance liquid chromatography (HPLC) were sulfuric acid (72%) (Fisher Scientific, Ricca Chemical). The chromatography standards used were D-(+)-cellobiose (Fluka), D-(+)-glucose (Sigma), D-(+)-xylose (Acros Organics), D-(−)-arabinose (Acros Organics), D-(+)-galactose (Acros Organics), D-(+)-mannose (Acros Organics), acetic acid (Sigma–Aldrich), 2-furfural dehyde (Fluka) and 5-hydroxymethyl-2-furfuraldehyde (HMF, Sigma–Aldrich Fine Chemicals). Methyl isobutyl ketone and ethanol used for digestion were from Fisher Scientific.

2.2. Experimental design

The experimental factors and corresponding values are presented in Table 1, whereas the experimental design is presented in Table 2. All experimental design runs were performed in duplicate. Response surface methodology (RSM) was used to design the experiment. A small central composite design (CCD) was used to study the impact of four factors: temperature, time, organic solvent mixture and acid concentration on responses such as glucose yield and delignification ratio. The experimental region included eight factorial design points, eight axial points ($\alpha = 1.41$) and five replicates of center points. The coded and the actual values of the four independent variables are presented in Table 2. Organic solvent mixture contained methyl isobutyl ketone (MIBK), ethanol (EtOH) and water in different proportions. Proportions of components (MIBK/EtOH/H_2O) in the ternary mixture were chosen based on the phase diagram (Solimo et al., 1989). Solvent ratios were chosen such that mixture remains as a single liquid phase (experimental solvent mixtures were chosen to be departed 5% from phase transition line into single phase region direction). Sulfuric acid (H_2SO_4) was used as a catalyst. The effects of temperature (104–146 °C), digestion time (16–44 min), MIBK content in organic solvent mixture (3–29% of MIBK) and quantity of catalyst (0.12–0.65%) were evaluated.

2.3. Clean fractionation (CF)

CF is a process of biomass fractionation using organic solvents and water. During this process, complex biomass is separated into three fractions: cellulose, hemicellulose and lignin. The simplified flowchart of the laboratory-scale CF process is presented in Fig. 1. The experimental setup was custom-made, and included stainless steel reactors and a metal heating block with six pockets. Each reactor (capacity ~ 250 mL) was equipped with a thermocouple, pressure gauge and quick connector attached to the cap of the reactor. These reactors were single-charge batch reactors that were sealed with a screw cap utilizing a Teflon ring as the seal. For each experiment, the same quantity of ground PCG and solvent were used: 7 g DM of PCG was loaded into the reactor and 100 g of organic solvent mixture with catalyst were added and sealed. During the digestion process the temperature was controlled by auto-tune temperature controllers (Omega, CN9121A) connected to a computer by a data acquisition device (Omega, OMB-DAQ-56). Temperature in each reactor was monitored by Lab View 8.2 software, and pressure was monitored by pressure gauge.

After the pulping process, the reactors were cooled to ambient temperature in a water bath, and subsequently the reaction mixture was unloaded and filtered through polypropylene cloth. Water (25 mL) was used to wash the reactor and "first wash" the solid fraction to remove residual MIBK. Both liquid fractions (filtrated liquor and water used to wash reactor and first wash of solid fraction) were collected and placed in a separatory funnel. "First wash" water added to the liquor induced the phase separation into organic and aqueous. The pulp remaining on polypropylene cloth contained mainly cellulose and was washed with additional water

Table 1
Experimental design factors and corresponding values.

Coded factors	Time(min)	Temperature(°C)	Catalyst – H_2SO_4(% w/w of solvent)	Solvent composition		
				MIBK (% w/w)	Water (% w/w)	Ethanol (% w/w)
$-\alpha$	16	104	0.12	3	26	71
−1	20	110	0.20	7	30	63
0	30	125	0.39	16	50	34
+1	40	140	0.57	25	35	40
$+\alpha$	44	146	0.65	29	35	36

Table 2
Central composite experimental design matrix.

Std.	Run	Actual				Coded			
		Factor 1A: time (min)	Factor 2B: temperature (°C)	Factor 3C: catalyst (% w/w)	Factor 4D: MIBK (% w/w)	Factor 1A: time (min)	Factor 2B: temperature (°C)	Factor 3C: catalyst (% w/w)	Factor 4D: MIBK (% w/w)
4	1	20	140	0.20	25	−1.000	1.000	−1.000	1.000
20	2[a]	30	125	0.39	16	0.000	0.000	0.000	0.000
1	3	40	140	0.57	7	1.000	1.000	1.000	−1.000
9	4	16	125	0.39	16	−1.414	0.000	0.000	0.000
14	5	30	125	0.65	16	0.000	0.000	1.414	0.000
5	6	40	110	0.20	25	1.000	−1.000	−1.000	1.000
19	7[a]	30	125	0.39	16	0.000	0.000	0.000	0.000
10	8	44	125	0.39	16	1.414	0.000	0.000	0.000
2	9	40	140	0.20	7	1.000	1.000	−1.000	−1.000
6	10	20	110	0.57	7	−1.000	−1.000	1.000	−1.000
13	11	30	125	0.12	16	0.000	0.000	−1.414	0.000
8	12	20	110	0.20	7	−1.000	−1.000	−1.000	−1.000
11	13	30	104	0.39	16	0.000	−1.414	0.000	0.000
12	14	30	146	0.39	16	0.000	1.414	0.000	0.000
16	15	30	125	0.39	29	0.000	0.000	0.000	1.414
18	16[a]	30	125	0.39	16	0.000	0.000	0.000	0.000
3	17	40	110	0.57	25	1.000	−1.000	1.000	1.000
15	18	30	125	0.39	3	0.000	0.000	0.000	−1.414
21	19[a]	30	125	0.39	16	0.000	0.000	0.000	0.000
17	20[a]	30	125	0.39	16	0.000	0.000	0.000	0.000
7	21	20	140	0.57	25	−1.000	1.000	1.000	1.000
Additional runs									
21	22	30	160	0.80	44	−1.000	2.333	2.243	3.111
22	23	30	160	0.57	7	−1.000	2.333	1.000	−1.000
23	24	30	140	0.80	7	−1.000	1.000	2.243	−1.000
24	25	30	140	0.57	44	−1.000	1.000	1.000	3.111

[a] Replicates of center points in experimental design.

(around 2 L) in order to remove MIBK residues. Washed cellulose solid fraction (output I) was transferred from the polypropylene cloth to a storage container, weighed and kept frozen for subsequent analysis and enzymatic hydrolysis. The liquor in the separatory funnel was kept for at least an hour in order to allow complete phase separation. Then the two phases, top organic (MIBK based) and the bottom aqueous were separated by releasing and collecting the bottom aqueous fraction with dissolved hemicellulose component (output II). Since the main component of the hemicellulose fraction is xylose (more than 75%), xylose measurements reflects degree of hemicellulose separation to aqueous fraction. The top fraction (output III), rich in lignin, was released from the separatory funnel and collected in another container. MIBK fraction was dried to remove the solvent, and the solid air-dried MIBK fraction was weighed.

2.4. Mass balance and fraction composition

Cellulose-, hemicellulose- and lignin-rich fractions were weighed to perform mass balance. The total solid (TS) content was measured in cellulose solid fraction in order to characterize the mass flow. Part of the cellulose fraction was dried in a convection oven at 45 °C, sugars and acid insoluble lignin (AIL) content in the cellulose fraction was also characterized. Compositional analyses of dried and ground cellulose samples were performed by acid hydrolysis according to NREL procedure NREL/TP-510-42618 (Sluiter et al., 2008c). The composition of the aqueous fraction, rich in hemicellulose components, was analyzed by direct injection of 10 μL into HPLC system (Agilent Technologies System 1200 with Bio-Rad Aminex 87H Column) with RID detector, 0.005 M H_2SO_4 mobile phase at a flow rate of 0.6 mL/min at 65 °C, as designated in NREL/TP-510-42623 (Sluiter et al., 2008b). One analysis ($n = 1$) was performed for each duplicate run.

2.5. Enzymatic hydrolysis of cellulose fraction

Enzymatic saccharification was employed in order to evaluate the efficiency of the pre-treatment. Hydrolysis was performed according to the NREL protocol described by Selig et al. in NREL/TP-510-42629 (Selig et al., 2008). For each experimental design run, cellulose fraction (output I obtained from CF process in Fig. 1) of 0.3 g of DM along with 0.1 M sodium citrate buffer with a pH of 4.8 were placed in scintillation vials. Hydrolysis was performed using cellulase and β-glucosidase enzymes, which were

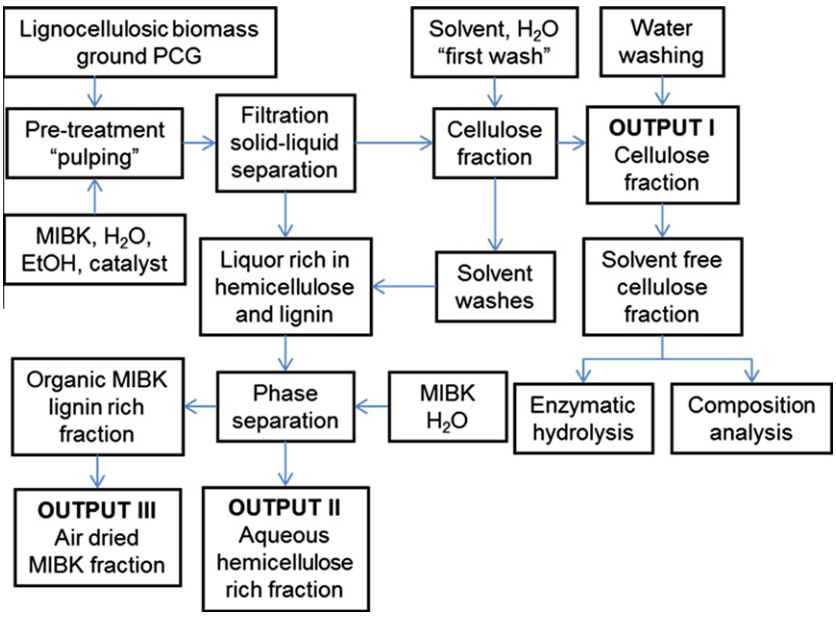

Fig. 1. Simplified flowchart of lab-scale clean fractionation (CF) process (adapted from Black et al. (1998)).

provided by Novozymes. The amount of cellulase (NS50013, activity 70 FPU/g, FPU = filter paper unit) enzyme was chosen to be 15 FPU/g DM, whereas β-glucosidase (NS50010, activity 250 CBU/g, CBU = cellobiase unit) dosage was 60 CBU/g DM. Hydrolysis was carried on for 72 h at 50 °C and 150 rpm in an incubated shaker (Fisher Thermo Scientific, Incubated Tabletop Orbital Shaker model 420). All hydrolysis runs were performed in duplicate. Samples of hydrolysates were collected and filtered through 0.2 μm nylon syringe filter. Concentrations of sugars, organic acids and by-products in the enzymatic hydrolysates were measured using HPLC system (Agilent Technologies System 1200 with Bio-Rad Aminex 87H Column) with RID detector, 0.005 M H_2SO_4 mobile phase at a flow rate of 0.6 mL/min at 65 °C and a sample volume of 20 μL, as designated by Sluiter et al. in NREL/TP-510-42623 (Sluiter et al., 2008b).

2.6. Statistical analysis

Statistical analyses were performed using Design Expert version 8.0.4. Multiple linear regression (MLR) method was used to determine model and response behavior. Statistical validation was performed by a one-way ANOVA test with 95% confidence (p-value <0.05).

3. Results and discussion

3.1. Mass balance

The mass balance of the CF process is presented in Table 3. As mentioned, the total solids content was measured in the cellulose solid fraction, while the rest of the initial DM input to the CF process was assumed to be solubilized in the aqueous and organic fractions. The yields of the pretreated material ranged between 35% and 80%. The solids recovery depended on the pre-treatment severity; solubilization of biomass components was higher with more severe pulping conditions, mainly associated with high levels of catalyst and temperature. Runs 3, 5, 14 and 21 resulted in relatively low pulp yield and were performed with either high level of temperature, high level of catalyst, or a combination of the two. On the contrary, run 12 resulted in high pulp yield since the independent variables were set at low values. A significant 2FI (two factorial interaction) regression model for pulp yield was created (based on a perturbation plot) with catalyst and temperature affecting pulp yield response the most. The graph showing pulp yield as a function of catalyst and temperature is presented in Fig. 2. The best fit regression model and resulting coefficients of determinations for pulp yield are presented in Table 4.

The highest amount of air-dried MIBK fraction corresponded to the highest amount of MIBK used for pulping. Runs 1, 17, 21 were performed with 25% MIBK, and run 15 with 28% MIBK in the pulping liquor, which resulted in the highest values of the air-dried MIBK fraction. Run 6, which was performed at the highest catalyst level (0.65%), also resulted in relatively high production of air-dried MIBK fraction. On the other hand, runs with low MIBK content resulted in low quantities of air-dried MIBK fractions. Runs 3, 9, 10, 12 (7% MIBK) and run 18 (3% MIBK) produced less than 0.21 g of air-dried MIBK fractions; also, run 13, carried out with low temperature (104 °C), resulted in a low air-dried MIBK fraction of 0.15 g. The highest amount of dried MIBK fraction was obtained for run 21, corresponding to the second lowest value of pulp yield (40%). For the optimal regression model for air-dried MIBK fraction yield, time was a non-significant factor, and was not included in the final terms of the model. A perturbation plot showed positive curvature on air-dried MIBK yield due to MIBK and linear positive effects due to temperature and catalyst factors. A response surface of air-dried MIBK fraction as a function of MIBK and catalyst quantity is presented in Fig. 2. The model equation and R-squared values are included in Table 4. In general, the lower the pulp yields the higher air-dried MIBK fraction output.

3.2. Fraction composition

Contents of glucose, xylose and lignin remaining in the cellulose fraction were analyzed using acid hydrolysis. Delignification extent was calculated by subtracting AIL remaining in the cellulose fraction after the CF process from the initial AIL content in the raw PCG material. Cellulose fraction characterization and delignification extent are presented in Table 5. The glucose remaining in the cellulose fraction ranged between 77% and 93%. The lowest amount of glucose remaining in the cellulose fraction corre-

Table 3
Mass balances of CF treatments (±1SD).

Std.	Run	PCG biomass DM[b] input (g)	Biomass DM[b] output in cellulose fraction (g)	Pulp yield (%)	Solubilized fraction of biomass in organic solvent mixture (g)	Air dried MIBK fraction (g)
4	1	7.00	4.38 ± 0.07	62.51	2.62	0.64 ± 0.08
20	2[a]	7.00	3.96 ± 0.14	56.52	3.04	0.48 ± 0.02
1	3	7.00	2.45 ± 0.14	34.97	4.55	0.21 ± 0.01
9	4	7.00	4.66 ± 0.23	66.63	2.34	0.39 ± 0.04
14	5	7.00	3.25 ± 0.03	46.47	3.75	0.66 ± 0.01
5	6	7.00	5.47 ± 0.03	78.18	1.53	0.43 ± 0.01
19	7[a]	7.00	4.29 ± 0.07	61.32	2.71	0.24 ± 0.00
10	8	7.00	3.59 ± 0.20	51.28	3.41	0.48 ± 0.02
2	9	7.00	4.54 ± 0.12	64.79	2.46	0.18 ± 0.01
6	10	7.00	5.15 ± 0.15	73.56	1.85	0.15 ± 0.03
13	11	7.00	5.24 ± 0.13	74.85	1.76	0.23 ± 0.04
8	12	7.00	5.58 ± 0.02	79.77	1.42	0.09 ± 0.01
11	13	7.00	5.49 ± 0.08	78.49	1.51	0.15 ± 0.04
12	14	7.00	2.83 ± 0.05	40.39	4.17	0.32 ± 0.05
16	15	7.00	3.72 ± 0.07	53.14	3.28	0.81 ± 0.05
18	16[a]	7.00	4.18 ± 0.01	59.75	2.82	0.43 ± 0.04
3	17	7.00	4.69 ± 0.08	66.97	2.31	0.90 ± 0.06
15	18	7.00	4.33 ± 0.06	61.82	2.67	0.14 ± 0.01
21	19[a]	7.00	4.04 ± 0.15	57.74	2.96	0.41 ± 0.08
17	20[a]	7.00	3.99 ± 0.08	56.94	3.01	0.40 ± 0.02
7	21	7.00	2.82 ± 0.06	40.27	4.18	1.02 ± 0.01
Additional runs						
21	22	7.00	2.04 ± 0.19	29.10	4.96	2.04 ± 0.11
22	23	7.00	2.77 ± 0.14	39.63	4.23	0.88 ± 0.07
23	24	7.00	2.88 ± 0.12	41.16	4.12	0.82 ± 0.01
24	25	7.00	2.71 ± 0.07	38.73	4.29	1.89 ± 0.01

[a] Replicates of center points in experimental design.
[b] DM denotes dry matter basis.

sponded to run 5, where the highest amount of catalyst was used (0.65%), while the highest amount of glucose remaining in the cellulose fraction occurred with runs 6 and 13; these were associated with a low value of temperature (run 13 with 104 °C), or a combination of low temperature and catalyst (run 6 with 110 °C and 0.2% catalyst).

The xylose remaining in the cellulose fraction spanned from 7% to 82%. The lowest values of xylose remaining in the cellulose fractions occurred in treatments with the lowest pulp yield (runs 3, 21, 14 and 5). These runs were performed with either high temperature level, high catalyst level, or a combination of the two. The highest values of xylose remaining in the cellulose fraction were observed for runs 6, 11, 12, 13, and were related to low levels of catalyst (run 6, 11, 12) and low temperature (run 13). Almost a linear relationship between glucose and xylose remaining in the cellulose fraction and two independent variables, catalyst and temperature, can be seen in Fig. 2. MIBK terms were insignificant for these regression models, while time played a less significant role than catalyst and temperature. Relationships between glucose and xylose remaining in the cellulose fraction and pulp yield were observed. Higher pulp yields resulted in more glucose and xylose remained in the pulp.

The highest amount of extracted AIL corresponded to run 3 (74%), 14 (73%) and 21 (79%). This behavior was related to high temperatures. Runs 3 and 21 were performed with high level catalyst (0.57%) and high level temperature (140 °C), while run 14 was performed at the highest initial experimental design temperature (146 °C). Low quantities of extracted AIL were correlated with low-temperature runs. Runs which resulted in less than 26% of extracted AIL were performed at 110 °C (runs 6, 10, 12) and 104 °C (run 13). Run 17, which was also conducted at 110 °C, resulted in relatively higher extracted AIL (34%); high levels of the three other processing factors (time, catalyst and MIBK content) were probably responsible for high response value (AIL). All of the lignin recoveries obtained in this process were considered relatively high, when compared to recent studies on organosolv treatment using wood as a feedstock (Garcia et al., 2011; Romani et al., 2011); however close to the results of studies using herbaceous feedstocks, e.g. sugarcane bagasse (Novo et al., 2011). A linear response surface for AIL extracted from the PCG biomass as a function of temperature and catalyst and quadratic response surface as a function of time and MIBK are depicted in Fig. 2. As presented in Fig. 3, the quantity of AIL extracted from PCG biomass decreased with an increase in pulp yield.

Aqueous fraction characteristics, including glucose and xylose content are presented in Table 5. Since the main component of the hemicellulose fraction is xylose (more than 75%), xylose measurements reflects degree of hemicellulose separation to aqueous fraction. Glucose leached to aqueous fraction comes from degradation of cellulose, and it is desired to keep it low, since it reduces glucose yield in enzymatic hydrolysis. Quantity of glucose extracted to the aqueous fraction was relatively consistent, and ranged between 7% and 10%. The highest amount of glucose extracted to the aqueous fraction corresponded to runs 3, 15 and 21. This behavior was associated with high levels of temperature and catalyst for runs 3 and 21. The model for glucose released to aqueous fraction was not statistically significant, however. More variation was observed for xylose released to aqueous fraction (than for glucose); its values ranged between 17% and 53%. The highest amount of xylose released to aqueous fraction corresponded to runs 3 and 21. The relatively high amount of xylose released to the aqueous fraction observed in runs 5, 14 and 15 could be the result of the highest value of catalyst, temperature and MIBK, respectively. A statistically significant model was built for xylose released to the aqueous fraction; however it was only based on two factors, temperature and catalyst. As indicated, runs 3 and 21 also resulted in the highest amount of extracted AIL (74% and 79%, respectively), lowest pulp yield (35% and 40%, respectively) and lowest xylose remaining in the cellulose fraction (7% and 13%). These relationships confirmed the expected outcome: runs with the lowest pulp

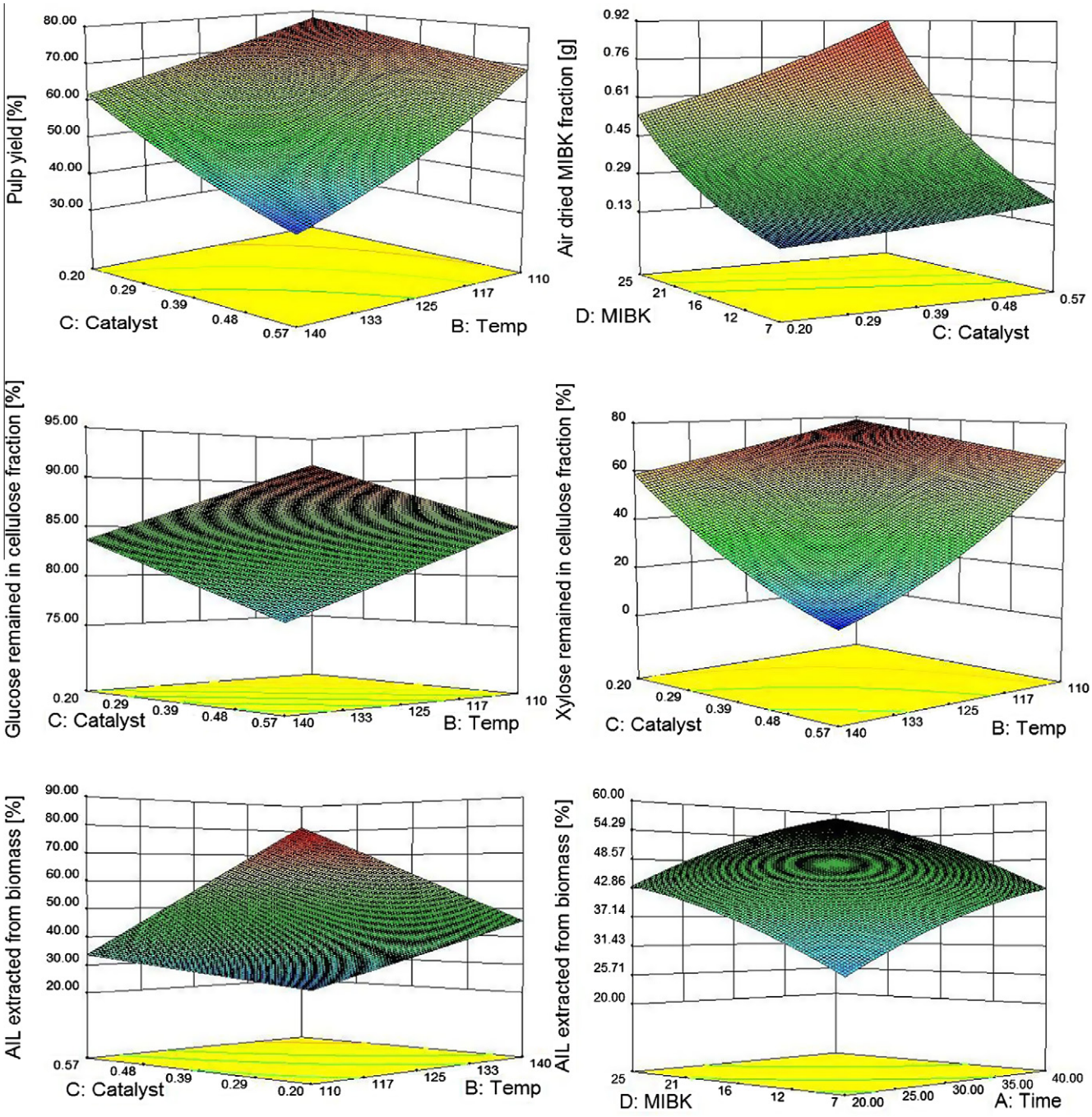

Fig. 2. Response surface plots of response variables (pulp yield, air dried MIBK fraction, glucose and xylose remained in cellulose fraction and AIL extracted from biomass) vs. significant factors.

yield resulted in the highest amount of components leached into the solvent mixture.

To create commercially competitive biorefineries, conversion of the entire lignocellulosic feedstock into marketable products must be pursued, so further investigation on the remaining two fractions (hemicellulose and lignin) is recommended. Purification, separation of its components and finding profitable application for hemicellulose and lignin streams would be the main aspects of future research. Also, overall economics of the organosolv process is strongly dependent on solvent recovery; therefore, recovery of solvents should also be taken into consideration and optimized.

3.3. Enzymatic hydrolysis of cellulose fraction

The yield of glucose was expressed as the percentage of glucose released in the enzymatic hydrolysate divided by the initial amount of glucose in the raw material. Hydrolysis efficiency was calculated as the percent of glucose submitted to enzymatic hydrolysis compared to the glucose that was enzymatically released. Glucose and xylose concentration in enzymatic hydrolysates as well as enzymatic hydrolysis glucose yield and enzymatic hydrolysis efficiency are presented in Table 6. Glucose concentrations in enzymatic hydrolysates were between 4 and 26 mg/mL. The

Table 4
Resulting regression models.

Regression model final equations in terms of actual factors,where A = time, B = temperature, C = catalyst, D = MIBK	R^2	Adjusted R^2	Predicted R^2
$\log_{10}$(pulp yield) = −1.02560 + 0.017771 ∗ A + 7.75550E−003 ∗ B + 1.84255 ∗ C + 0.023633 ∗ D − 1.51679E−004 ∗ AB − 7.35971E−003 ∗ AC − 0.016052 ∗ BC − 2.09703E−003 ∗ BD	0.9827	0.9712	0.9038
$\log_{10}$(air dried D fraction) = −2.0442 + 6.2696E−003 ∗ B + 0.6401 ∗ C + 0.0344 ∗ D	0.8680	0.8448	0.8066
Glucose remaining in the cellulose fraction = 118.82997 + 0.26611 ∗ A − 0.28068 ∗ B + 11.63424 ∗ C − 1.03263 ∗ AC	0.8280	0.7850	0.6605
(Xylose remaining in the cellulose fraction)$^{1/2}$ = 6.83143 − 0.026786 ∗ A + 0.029859 ∗ B + 36.13067 ∗ C − 0.34848 ∗ B ∗ C	0.9533	0.9416	0.9162
AIL extracted from PCG biomass = −1.85862 ∗ A − 1.54869 ∗ B − 258.14319 ∗ C − 4.51450 ∗ D + 0.030393 ∗ A ∗ B + 2.53608 ∗ B ∗ C + 0.049157 ∗ B ∗ D − 0.019213 ∗ A^2 − 0.022773 ∗ D^2	0.9894	0.9807	0.8342
1/(Xylose released to aqueous fraction)$^{1/2}$ = −0.46855 + 0.010535 ∗ B + 0.84418 ∗ C − 7.81186E−003 ∗ B ∗ C − 3.68937E−005 ∗ B^2	0.8693	0.8366	0.8061
Enzymatic hydr. glucose yield = + 8.34142 + 0.16655 ∗ A + 0.08161 ∗ B − 213.06315 ∗ C + 2.26510 ∗ B ∗ C	0.9679	0.9598	0.9538
Logit(enzymatic hydr. efficiency) = ln[(enzymatic hydr. efficiency + 0.00)/(100.00 − Enzymatic hydr. efficiency)] = +20.56275 + 0.013246 ∗ A − 0.31490 ∗ B − 37.04755 ∗ C + 0.30882 ∗ B ∗ C + 1.11047E−003 ∗ B^2 + 5.93691 ∗ C^2	0.9861	0.9802	0.9703
Models with extra runs			
1/(Enzymatic hydr. glucose yield)$^{1/2}$ = +0.81067 − 3.64465E−004 ∗ A − 8.24894E−003 ∗ B − 0.23839 ∗ C + 2.78021E−005 ∗ B^2 + 0.18594 ∗ C^2	0.9384	0.9222	0.8886
Logit(AIL extracted from PCG biomass) = ln[(AIL extracted from biomass + 0.00)/(100.00 − AIL extracted from biomass)] = −4.35454 − 0.11869 ∗ A + 0.014041 ∗ B + 5.00937 ∗ C + 0.028493 ∗ D + 1.08629E−003 ∗ A ∗ B − 3.21554 ∗ C^2	0.9309	0.9079	0.8106

highest glucose concentrations corresponded to runs 3 (26 mg/mL), 21 (23 mg/mL), 14 (20 mg/mL) and 5 (16.65 mg/mL). These runs also showed the highest glucose yield and enzymatic hydrolysis efficiency. The same runs also resulted in the highest xylose concentration in the enzymatic hydrolysates. As an example, enzymatic hydrolysis reaction rate for run 21 (run with the highest enzymatic hydrolysis glucose yield) is presented in Fig. 3. The concentration of glucose stabilized after 48 h, indicating that enzymatic hydrolysis was complete after 48 h. Glucose yield reached 83.86%, 82.15%, 72.59%, 68.94%, and enzymatic hydrolysis efficiency was 98.41%, 98.72%, 92.12% and 89.93% in runs 21, 3, 14 and 5, respectively. Obtained glucose yields were found comparable to a recent study utilizing glycerol as a pulping agent (Novo et al., 2011) and can be considered relatively high when compared to a two-step process utilizing acetic acid pulping of *Eucalyptus globulus* wood as a feedstock (Romani et al., 2011). The same runs produced the lowest pulp yield caused by either high temperature level, high catalyst level, or a combination of the two. Similar trend was observed by Novo et al. (2011), where applying high temperature resulted in low pulp yields due to high delignification rates, high hemicellulose removal and partial cellulose dissolving. The lowest enzymatic hydrolysis yield and efficiency was observed for runs 6, 11, 12, 13. The low yield was due to low catalyst level (runs 6, 11, 12) and low temperature level (run 13). These runs also resulted in the highest amount of xylose and AIL remaining in the cellulose fraction. High amount of lignin and xylose remaining in the cellulose fraction resulted in low glucose yield and low enzymatic hydrolysis efficiency.

As reported by various researchers, mechanisms and interactions of cellulolytic enzymes are affected by both substrate structure and lignin, which is one of the most limiting factors for enzyme action (Hsu, 1996). Lignin is largely responsible for unproductive binding to cellulase enzymes, which restricts the enzymatic hydrolysis of cellulose (Hetti, 2004). Chernoglazov et al. observed that endoglucanases lost activity when sorbed onto lignin (Chernoglazov et al., 1988). Results from our study indicate that delignification is indeed an important aspect of efficient pre-treatment of lignocellulosic biomass, especially for PCG.

Presence of hemicellulose is also a determining factor of enzymatic hydrolysis glucose yield and efficiency. Hemicellulose is in close association with cellulose, therefore removal of hemicellulose will result in cellulose that is more susceptible to enzyme attack. As presented in Fig. 3, there was a negative correlation between xylose remaining in the cellulose fraction and glucose yield. In related work, lignocellulosic materials were pretreated with sulfuric acid and a negative relation to hemicellulose remaining and enzyme digestibility was found (Grohmann et al., 1985; Knappert et al., 1981). Another type of enzyme inactivation and unproductive binding, or entrapment, of cellulases in the small pores of cellulose has been suggested by some researchers (e.g. studies on steam-exploded *Pinus radiata* showed a relationship between fiber porosity and cellulose digestibility). In this study investigators have also shown that the removal of hemicellulose and lignin structure reorganization increased the surface area present in the form of pores and has resulted in increased accessibility to enzymes (Wong et al., 1988). Thus more detailed characterization of the cellulose fraction in our study would be warranted to address significance of mentioned parameters on cellulose digestibility.

Enzymatic hydrolysis glucose yield and efficiency models were generated and are presented in Fig. 3. A 2FI model for glucose yield was built, with time, temperature and catalyst as linear terms, and a temperature–catalyst cross-product as the interaction term. MIBK factor was not significant for this model. Regression models and R^2 values for enzymatic hydrolysis glucose yield and efficiency are presented in Table 4.

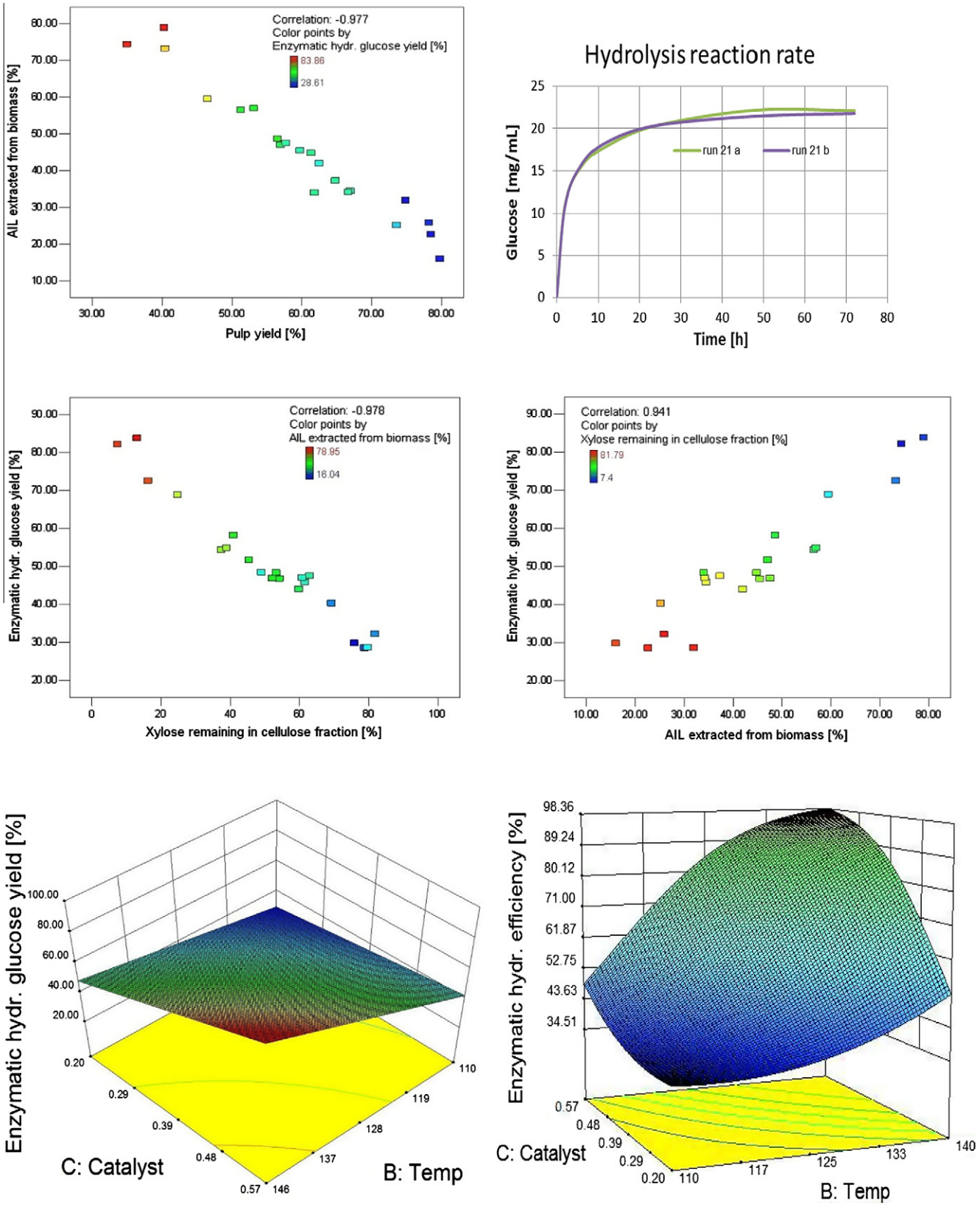

Fig. 3. Significant correlations between AIL extracted from biomass and pulp yield, enzymatic hydrolysis glucose yield and xylose remaining in cellulose fraction and AIL extracted from biomass, and enzymatic hydrolysis characteristics (response surface plots and hydrolysis reaction rate).

A relationship between enzymatic hydrolysis glucose yield and the AIL remaining in the cellulose fraction was observed. The glucose yield increased with delignification extent. Clean fractionated cellulose samples with a small quantity of AIL resulted in high glucose yield, as presented in Fig. 3. In related work it was reported that the effect of xylan hydrolysis and its removal in pre-treatment

Table 5
Glucose, xylose and lignin content in the cellulose fraction and aqueous fractions (±1SD).

Std.	Run	Glucose remaining in the cellulose fraction (%)[b]	Xylose remaining in the cellulose fraction (%)[c]	AIL remaining in the cellulose fraction (%)[d]	AIL extracted from PCG biomass (%)[d]	Glucose released to aqueous fraction (%)[b]	Xylose released to aqueous fraction (%)[c]
4	1	84.20 ± 1.90	59.78 ± 2.30	57.98 ± 1.26	42.02 ± 1.26	7.10 ± 0.12	17.34 ± 0.28
20	2[a]	84.08 ± 1.06	40.92 ± 4.43	51.37 ± 3.41	48.63 ± 3.41	6.80 ± 0.10	19.34 ± 1.41
1	3	85.00 ± 4.39	7.40 ± 0.07	25.59 ± 1.62	74.41 ± 1.62	10.15 ± 0.65	52.74 ± 2.06
9	4	87.22 ± 1.36	60.74 ± 7.30	65.80 ± 3.11	34.20 ± 3.11	6.78 ± 0.09	17.12 ± 1.04
14	5	76.67 ± 0.45	24.79 ± 0.18	34.26 ± 0.05	65.74 ± 0.05	7.18 ± 0.25	31.88 ± 2.01
5	6	92.64 ± 1.32	81.79 ± 2.25	74.07 ± 1.42	25.93 ± 1.42	7.55 ± 0.13	17.14 ± 0.32
19	7[a]	87.74 ± 2.92	53.29 ± 4.27	55.18 ± 3.28	44.82 ± 3.28	7.36 ± 0.05	20.07 ± 0.38
10	8	78.54 ± 3.45	37.39 ± 1.36	43.52 ± 1.48	56.48 ± 1.48	7.26 ± 0.03	21.43 ± 0.61
2	9	86.30 ± 0.07	62.89 ± 4.61	62.68 ± 2.33	37.32 ± 2.33	7.48 ± 0.40	17.53 ± 1.43
6	10	86.79 ± 0.37	69.23 ± 0.06	74.80 ± 0.87	25.20 ± 0.87	8.34 ± 0.51	19.60 ± 1.28
13	11	87.68 ± 2.43	79.66 ± 1.91	68.05 ± 1.42	31.95 ± 1.42	8.19 ± 0.06	18.32 ± 0.42
8	12	88.85 ± 1.39	75.88 ± 1.74	83.96 ± 0.06	16.04 ± 0.06	7.66 ± 0.41	17.04 ± 0.96
11	13	92.53 ± 1.09	78.76 ± 1.65	77.38 ± 1.35	22.62 ± 1.35	7.52 ± 0.06	16.69 ± 0.39
12	14	78.79 ± 2.17	16.23 ± 1.85	26.72 ± 2.43	73.28 ± 2.43	7.96 ± 0.51	39.90 ± 3.47
16	15	83.04 ± 1.06	38.95 ± 3.25	43.04 ± 2.12	56.96 ± 2.12	9.08 ± 0.25	27.51 ± 1.08
18	16[a]	83.55 ± 0.90	54.26 ± 0.90	54.51 ± 0.36	45.49 ± 0.36	7.43 ± 0.36	19.51 ± 0.30
3	17	85.58 ± 1.11	61.57 ± 2.68	65.51 ± 0.22	34.49 ± 0.22	7.26 ± 0.10	17.70 ± 0.28
15	18	85.43 ± 1.32	48.99 ± 3.12	65.98 ± 0.75	34.02 ± 0.75	7.29 ± 0.02	21.40 ± 0.13
21	19[a]	81.93 ± 2.14	52.20 ± 7.75	52.44 ± 4.43	47.56 ± 4.43	7.07 ± 0.08	18.85 ± 0.89
17	20[a]	86.08 ± 1.91	45.38 ± 5.42	52.96 ± 3.62	47.04 ± 3.62	6.37 ± 0.22	17.11 ± 1.01
7	21	85.22 ± 3.92	13.09 ± 0.12	21.05 ± 0.87	78.95 ± 0.87	9.06 ± 0.01	45.87 ± 1.00
21	22	67.76 ± 4.12	0.00 ± 0.00	13.14 ± 3.31	86.86 ± 3.31	6.33 ± 0.73	23.53 ± 0.41
22	23	80.57 ± 2.20	0.07 ± 0.10	29.64 ± 8.96	64.02 ± 8.96	12.69 ± 0.89	58.39 ± 5.90
23	24	79.59 ± 2.09	9.35 ± 1.15	33.01 ± 2.57	66.99 ± 2.57	11.53 ± 0.37	60.76 ± 0.74
24	25	81.21 ± 0.61	5.72 ± 0.07	20.50 ± 0.27	79.50 ± 0.27	6.48 ± 0.46	30.03 ± 1.19

[a] Replicates of center points in experimental design.
[b] Percent of initial glucose content in raw PCG biomass.
[c] Percent of initial xylose content in raw PCG biomass.
[d] Percent of initial AIL content in raw PCG biomass (AIL = acid insoluble lignin).

of corn stover was more significant than the effect of lignin removal for improving the digestibility and accessibility of cellulose. However, it was also found that the removal of xylan and lignin may cause aggregation of the cellulose microfibrils, resulting in hindrance of enzyme accessibility (Ishizawa et al., 2009). In addition to lignin content and distribution, there are other structural properties potentially limiting to enzymatic hydrolysis of cellulosic fibers, such as degree of polymerization, crystallinity, cellulose lattice structure, structural composition, particle size, fiber dimensions, available surface area and a degree of fiber swelling, pore structure and distribution (Mansfield et al., 1999). A detailed study of these parameters would be highly recommended in future research, for better understanding of the enzymatic hydrolysis process of clean fractionated cellulose samples.

3.4. Optimization

Several of the models presented in Table 4 were subjected to transformation processes in order to improve the statistical analysis and/or diagnostic plots. A Box-Cox plot tool was used to determine the most appropriate power transformation to apply to the response data. The lowest point on the Box-Cox plot suggests transformed model with minimum residual sum of squares. Because R^2 can be artificially inflated by adding terms to the model, the adjusted and predicted R^2 are presented as well. A rule of thumb is that the adjusted and predicted R^2 values should be within 0.2 of each other, which is the case in our models (Anderson and Whitcomb, 2004).

The main intent of this study was to find the highest glucose yield produced by enzymatic hydrolysis. Based on the numerical optimization of glucose yield, the highest value was 84%, which corresponded to 30 min, 140 °C, 0.57% catalyst and 7% MIBK. With this criteria, AIL extracted from the PCG biomass was equal to 66% and xylose released to aqueous fraction was 50%. On the other hand, if the goal was to find the highest glucose yield (85%) together with maximizing the amount of AIL extracted from the PCG (93%) and xylose released to aqueous fraction (50%), the conditions are 40 min,140 °C, 0.57% catalyst and 17% MIBK. Using these latter conditions AIL extracted from PCG biomass would be higher by 27%, as a result of increasing MIBK by 10% and treatment time by 10 min. The models presented for glucose yield and AIL extracted from PCG biomass illustrate the effect of factors on the response surface; however, no critical value of the response surface was observed for these two models (Fig. 3).

Since RSM is a modeling technique with the purpose of locating an optimum value, four additional runs were carried out and the values of the factors were an extension of the operability region from the point with the highest glucose yield (140 °C, 0.57% catalyst, 7% MIBK and 30 min) and are presented in Table 2. Three factors were expanded except for time. As presented in Table 5 time term coefficient in enzymatic hydrolysis glucose yield model was low therefore had little effect on this response. Temperature was extended to 160 °C, catalyst to 0.8% and MIBK content to 44%. The results of pulp yield, dried MIBK fraction, glucose, xylose and AIL remaining in the cellulose fraction, glucose and xylose released to the aqueous fraction, and glucose yield and enzymatic hydrolysis efficiency are all presented in Tables 3, 5 and 6, respectively. Lowest pulp yield (29%) was observed for run 22 where all the factor levels were increased except time. Air dried MIBK fraction increased to 2 and 1.9 g in runs 22 and 25, respectively; this was associated with higher MIBK content (44%) in these runs. Glucose remaining in the cellulose fraction reached the lowest value (68%) for run 22. Xylose remaining in the cellulose fraction went to 0 for runs 22 and 23, but for runs 24 and 25 had a value of 9% and 5%, respectively. Complete removal of xylose from the cellulose fractions in runs 22 and 23 did not result in complete recovery of xylose in aqueous fraction. It can be suspected that it was due to transfer of xylose to organic fraction or incomplete depolymerization of hemicellulose to xylose. AIL extracted from PCG biomass reached 86% for run 22, which was 8% higher than for run 21 in

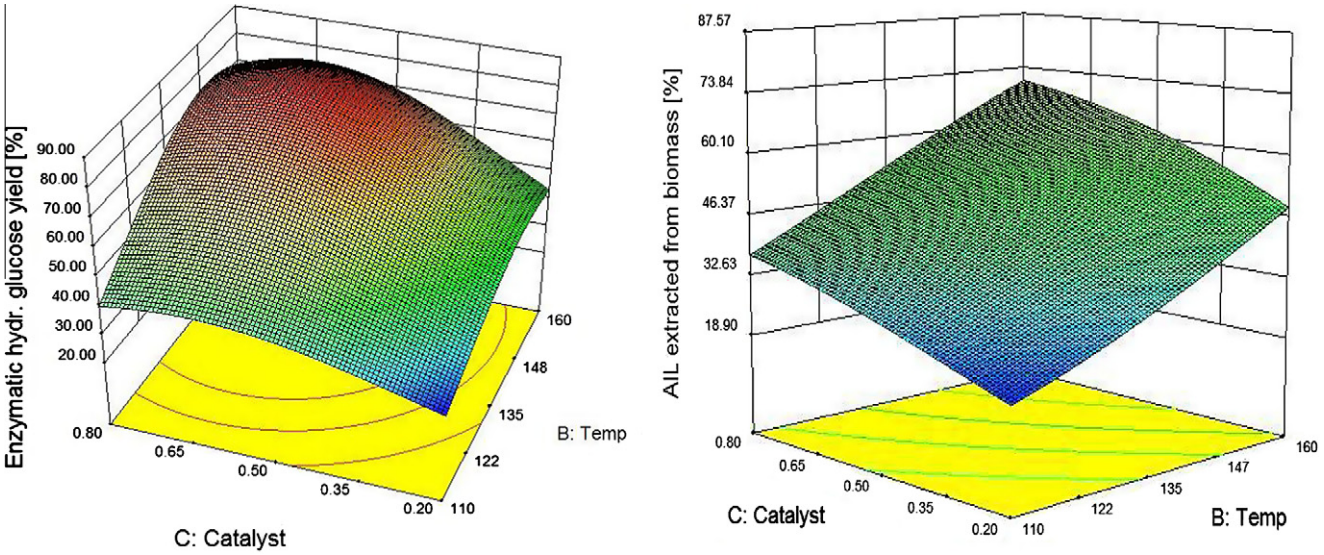

Fig. 4. Effect of catalyst (% w/w) and temperature (°C) on enzymatic hydrolysis glucose yield (%) and AIL extracted (%) from PCG biomass (model with extra runs).

Table 6
Results of enzymatic hydrolysis of the cellulose fraction (±1SD).

Std.	Run	Glucose concentration in enzymatic hydrolysate (mg/mL)	Xylose concentration in enzymatic hydrolysate (mg/mL)	Enzymatic hydrolysis glucose yield (%)	Enzymatic hydrolysis efficiency (%)
4	1	7.91 ± 0.13	2.16 ± 0.01	44.05 ± 0.01	52.32
20	2[a]	11.58 ± 1.66	2.71 ± 0.24	58.20 ± 6.23	69.21
1	3	26.38 ± 0.50	3.32 ± 0.18	82.15 ± 3.24	98.72
9	4	7.93 ± 0.90	1.77 ± 0.20	46.98 ± 3.03	53.87
14	5	16.65 ± 0.23	3.37 ± 0.01	68.94 ± 1.56	89.93
5	6	4.61 ± 0.40	0.70 ± 0.15	32.15 ± 3.02	34.71
19	7[a]	8.84 ± 0.19	2.02 ± 0.00	48.30 ± 0.26	55.05
10	8	11.89 ± 0.11	2.75 ± 0.10	54.30 ± 2.60	69.15
2	9	8.23 ± 0.61	2.08 ± 0.03	47.50 ± 2.24	55.04
6	10	6.15 ± 0.34	1.31 ± 0.23	40.30 ± 1.07	46.43
13	11	4.30 ± 0.17	0.61 ± 0.00	28.69 ± 0.42	32.73
8	12	4.19 ± 0.31	0.54 ± 0.05	29.80 ± 2.11	33.54
11	13	4.09 ± 0.04	0.63 ± 0.03	28.61 ± 0.14	30.92
12	14	20.17 ± 1.03	3.34 ± 0.03	72.59 ± 2.43	92.12
16	15	11.59 ± 0.89	3.00 ± 0.09	54.87 ± 3.11	66.08
18	16[a]	8.78 ± 0.31	2.11 ± 0.16	46.75 ± 1.56	55.95
3	17	7.68 ± 0.05	2.06 ± 0.06	45.84 ± 0.45	53.56
15	18	8.79 ± 0.73	1.83 ± 0.19	48.42 ± 3.34	56.68
21	19[a]	9.14 ± 1.66	2.20 ± 0.40	46.88 ± 6.74	57.22
17	20[a]	10.19 ± 0.43	2.49 ± 0.05	51.70 ± 1.12	60.06
7	21	23.36 ± 0.65	3.72 ± 0.07	83.86 ± 4.08	98.41
Additional runs					
21	22	28.00 ± 1.26	0.25 ± 0.17	62.86 ± 1.64	92.77
22	23	22.90 ± 1.00	1.37 ± 0.34	67.51 ± 5.07	83.79
23	24	20.53 ± 1.26	2.52 ± 0.08	66.19 ± 1.96	83.17
24	25	22.39 ± 0.63	2.38 ± 0.04	68.75 ± 0.22	84.66

[a] Replicates of center points in experimental design.

the original design. Glucose and xylose released to the aqueous fraction was the highest for runs 23 and 24. Highest glucose concentration in the enzymatic hydrolysate was observed for run 22 (28 mg/mL). However, because of the lowest pulp yield in this run it did not give the highest glucose yield. Enzymatic hydrolysis glucose yield was between 63% and 69%, which was lower than in the original design. A similar reduction was observed for enzymatic hydrolysis efficiency in the additional runs (83–93%). The regression model for glucose yield with extra design points reached a critical value in the surface presented in Fig. 4. A quadratic model was generated, and again the MIBK factor was not significant, as shown in Table 4. The response surface graph for AIL extracted from PCG biomass is presented in Fig. 4, and the corresponding regression model equation is included in Table 4. No surface critical value was obtained for this model, however.

Based on numerical optimization for the experimental design, including the additional points, the highest response for glucose yield was 84%, and corresponded to 38 min, 143 °C, 0.65% catalyst and 8% MIBK. With the same pretreatment conditions, AIL extracted from PCG reached 77%. If the highest glucose yield (84%) and the highest AIL extracted from PCG (91%) was of interest, the processing conditions would be 39 min, 152 °C, 0.69% catalyst and 29% MIBK. The additional 14% AIL extracted from PCG did not improve glucose yield, and required higher temperature, more catalyst and significantly more MIBK (21% more). An alternative solution suggested by numerical optimization was 84% glucose

yield and 87% AIL extracted from PCG biomass, corresponding to 39 min, 154 °C, 0.69% catalyst and 9% MIBK. These conditions seem to be the most preferable if minimal consumption of MIBK was desired; however, specific applications for hemicellulose and lignin fraction would have to be proposed and economic analysis would have to be included in order to say which conditions are more profitable.

4. Conclusion

RSM was successfully applied to optimize time, temperature, catalyst dosage and solvent composition on glucose yield response. No critical value for the AIL extracted from PCG biomass response surface was found. Optimal conditions resulted in 84% glucose yield and 87% of AIL extracted from PCG, corresponding to 39 min, 154 °C, 0.69% catalyst and 9% MIBK content. Negative correlations between xylose and AIL remaining in the cellulose fraction and with glucose yield were found. Catalyst and temperature were the most important factors for all the models. Quantity of MIBK was significant for AIL extracted, pulp yield and air-dried MIBK fraction models.

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