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Enhanced Enzymatic Saccharification of Barley Straw Pretreated by Ethanosolv Technology

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Abstract The fermentable sugars in lignocellulosic biomass are derived from cellulose and hemicellulose, which are not readily accessible to enzymatic saccharification because of their recalcitrance. An ethanosolv pretreatment method was applied for the enzymatic saccharification of barley straw with an inorganic acid. The effects of four process variables (temperature, time, catalyst dose, and ethanol concentration) on the barley straw pretreatment were analyzed over a broad range using a small composite design and a response surface methodology. The yield of the residual solid and composition of the solid fraction differed as ethanosolv conditions varied within the experimental range. A glucan recovery, xylan recovery, and delignification were 85%, 14%, and 69% at center point conditions (170°C, 60 min, 1.0% (*w*/*w*) H_2SO_4, and 50% (*w*/*w*) ethanol), respectively. Ethanosolv pretreatment removed lignin effectively. Additionally, the highest enzymatic digestibility of 85.3% was obtained after 72 h at center point conditions.

Keywords Organosolv pretreatment · Ethanosolv · Lignocellulose · Barley straw · Bioethanol

Introduction

Bioethanol can be produced from a lignocellulosic material using a pretreatment followed by enzymatic hydrolysis and fermentation [1–3]. Obtaining a high overall ethanol yield depends on the pretreatment method [4]. An effective pretreatment disrupts the cell wall as well as the cellulose crystallinity so that the hydrolytic enzymes can access the biomass macrostructure [5–7]. Additionally, the pretreatment affects the cost of the other operations, including the size reduction prior to the pretreatment and enzymatic hydrolysis after the

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pretreatment. Pretreatment can also strongly influence the downstream costs by impacting the fermentation toxicity, enzymatic hydrolysis rate, enzyme loading, and other process variables [8].

Various pretreatment methods have been developed, but only some of them seem promising. These pretreatments include physical (comminution, hydrothermolysis), chemical (acid, alkali, solvents, ozone), physico-chemical (steam explosion, ammonia fiber explosion), and biological pretreatment techniques that have been developed to improve the accessibility of the enzymes to the cellulosic fibers.

The organosolv process has been investigated for paper production to extract lignin from the pulp. In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl, H_2SO_4) is used to break the lignin and hemicellulose bonds. The organic solvents used in the process are methanol, ethanol, acetone, ethylene glycol, triethylene glycol, and tetrahydrofurfuryl alcohol [6]. Alcohols, especially primary alcohols such as methanol and ethanol, are the most frequently used solvents. Methanol and ethanol seem to be the most favored alcohols for alcohol-based organosolv pretreatment because of their low cost and ease of recovery [6]. The ethanol pretreatment is safer than the methanol pretreatment because ethanol is less toxic than methanol. Ethanol can also be easily recovered through distillation. To date, the ethanol organosolv pretreatment has been applied to woody biomass. This study attempted to apply the ethanol organosolv pretreatment to the crop residue of barley.

Barley straw is considered to account for large portion of available biomass feedstock in Korea (e.g., 2.25×10^5 t of dry barley straw per year, 2006), and Honam district is responsible for 70% of the annual production. Barley straw was burned on site after harvesting, which causes air pollution. So barley straw could be obtained as byproduct from agriculture and promise for use as feedstock for the production of fermentable sugars.

In this study, the ethanol organosolv pretreatment was named “ethanosolv”. The objectives of this study were to examine the influence of various process parameters on the treatment of the barley straw and the enzymatic digestibility and to determine the specific relationship of the enzymatic digestibility.

Materials and Methods

Lignocellulose and Solvents

Barley straw (harvested in 2008) was obtained from the Honam Agricultural Research Institute, Korea. The air-dried barley straw was then milled using a cutting mill (Sungchang Co. Ltd., Korea) and stored in sealed plastic bags at room temperature. Avicel (Merck, USA) was used as pure cellulose. Ethanol, H_2SO_4, and other chemicals used in this study were purchased from Junsei, Japan. All of these chemicals were guaranteed reagent grade and were used without further purification.

Analytical Method

The composition of the barley straw was analyzed by the National Renewable Energy Laboratory (NREL) LAP-002 procedure to evaluate the carbohydrates in the biomass [9] and by the NREL LAP-003, 004 procedure to evaluate the acid-insoluble lignin and the acid-soluble lignin [10, 11].

The carbohydrate contents of the untreated and pretreated barley straw were determined by measuring the hemicellulose (xylose, galactose, and arabinose) and cellulose-derived sugars (glucose) using high-performance liquid chromatography (HPLC). Samples were filtered through syringe microfiltration (0.2 μm). The HPLC system was equipped with a Bio-Rad Aminex HPX-87P column, a guard column, an automated sampler, a gradient pump, and a refractive index detector. The mobile phase was deionized water at a flow rate of 0.6 ml/min at 85°C.

Ethanosolv Pretreatment

Samples (6 g) of grounded barley straw in 8–92% (*w*/*w*) aqueous ethanol with a 0.16–1.84% (*w*/*w*) H_2SO_4 solution were heated at 136–204°C for 26–94 min. The ethanosolv liquor was prepared by 95% ethanol, 72% H_2SO_4, and distilled water for experimental concentrations. The ratio of liquor-to-barley straw was constant (7:1 *v*/*w*; 42 ml:6 g) in all experiments. The ethanosolv experiments were performed in a 100-ml Teflon vessel Φ 47×93 mm with wall thickness of 0.4 mm using an oil bath. After ethanosolv, the vessels were cooled in a water bath. The residual solid and liquor were then separated through microfiltration (0.20 μm, Nylon). The residual solid was then washed with 100 ml aqueous ethanol and the same concentration of ethanosolv liquor. The washed residual solid was dried at 45°C for 24 h and then used for enzymatic hydrolysis.

Enzymatic Hydrolysis

Enzymatic hydrolysis of the barley straw was conducted with cellulase (Celluclast 1.5 L, Sigma, USA) and β-glucosidase (Novozyme 188, Sigma, USA) following the NREL LAP-009 procedure [12]. Cellulase was supplemented with β-glucosidase (1 FPU:2 CBU) to avoid product inhibition caused by cellobiose accumulation [13]. Cellulase and β-glucosidase were used at enzyme loadings of 20 FPU/g cellulose and 40 CBU/g cellulose, respectively. Enzymatic hydrolysis was performed at 45°C with a rotational speed of 150 rpm for 72 h in a rotary shaker, and samples were periodically taken to determine the glucose concentration. The enzymatic digestibility was expressed as the amount of glucose produced to the total amount of glucose in the initial biomass.

Results and Discussion

Characteristics of Barley Straw

The chemical composition of the barley straw varies depending on the growing location, season, harvesting methods, as well as the analysis procedures. The composition of the barley straw used in this study is presented in Table 1. The sugar fraction of the dry biomass was 60.71% based on the HPLC carbohydrate analysis. Glucan was the major component at 38.08%, and xylan, which was the major hemicellulose constituent, was 18.74%. Arabinan accounted for only a small portion of the biomass, while galactan and mannan were not detected. These compounds can be converted to ethanol by a pentose- and hexose-fermenting organisms. However, fermentation of pentose with a microorganism was too difficult, so in this study, cellulose was retained in the solid during pretreatment and then utilized using the fermentation of hexose derived from the solid portions.

Table 1 Composition of the untreated barley straw.

Component	Percentage (%)[a]
Cellulose	
Glucan	38.08
Hemicellulose	
Xylan	18.74
Arabinan	3.89
Galactan	–
Mannan	–
Lignin	
Acid-soluble lignin	1.82
Acid-insoluble lignin	20.45
Ash	4.22

[a] Composition percentages are on a dry-weight basis.

Effects of Ethanosolv Pretreatment on the Composition of the Solid Fraction

The influence of four process variables (temperature, time, H_2SO_4 concentration, and ethanol concentration) on the yield of the residual solid and the compositions of the solid fraction were investigated by response surface method using a small composite design analysis. The composite design comprised 21sets of conditions, including eight factorial points, eight star points, and five center points, as summarized in Table 2. The range of conditions examined were temperature 136–204°C; time 26–94 min; H_2SO_4 0.16–1.84% (*w*/*w*); and ethanol 8–92% (*w*/*w*). The center point (Table 2, nos. 17–21) conditions (170°C; 60 min; 1.0% (*w*/*w*) H_2SO_4 and 50% (*w*/*w*) ethanol) were selected on the basis of the results from preliminary tests (data not shown). Regression analysis using the SigmaPlot® software version 10.0 (Systat software Inc., Germany) produced a quadratic equation that was used to model the effect of each process variable on the responses (residual solid, glucan, xylan, lignin, enzymatic digestibility). The equations determined for each response are listed in Table 3. The yield of the residual solid and the composition of the solid fraction differed as the ethanosolv conditions within the experimental range.

Figure 1 shows the effects of single process parameters, while the others were fixed using the response surface methodology. The yields of the residual solid, glucan, and xylan decreased as the ethanosolv temperature increased within the experimental range. The reduced residual solid was largely attributed to the accelerated solubilization and degradation of the cellulosic and hemicellulosic sugars. This explanation was consistent with the general decline in the glucan and xylan levels. Additionally, all the arabinan was solubilized and degraded. The yield of lignin was lowest at 150°C and increased greatly above 170°C to a maximum at 204°C, which was probably due to the lignin condensation reaction at higher temperatures.

Most of the responses showed a linear time-dependence except for glucan. The residual solid, xylan, and lignin yields decreased with longer ethanosolv times. Lower levels of lignin following extended ethanosolv times indicated that the lignin degradation increased during the ethanosolv pretreatment. The yield of glucan was insensitive to the ethanosolv time.

Table 2 Description of the ethanosolv pretreatment.

No.	Temperature (°C)	Time (min)	Sulfuric acid concentration (%)	Ethanol concentration (%)	Residual solid (%)	Yield			Enzymatic digestibility (%)
						Glucan (%)	Xylan (%)	Lignin (%)	
1	190	80	1.5	25	32.66	0.80	0.14	22.20	–
2	190	80	0.5	25	54.76	36.06	1.33	16.99	80.99
3	190	40	1.5	75	37.86	24.14	0.61	4.54	82.31
4	150	80	0.5	75	70.80	35.96	8.98	9.88	59.20
5	190	40	0.5	75	66.76	35.64	9.08	9.19	78.58
6	150	40	1.5	25	59.60	35.37	6.11	14.55	71.42
7	150	80	1.5	75	54.27	31.86	4.58	5.90	83.96
8	150	40	0.5	25	87.69	38.07	14.35	21.66	47.66
9	136	60	1.0	50	62.64	36.15	8.63	9.89	68.73
10	204	60	1.0	50	28.79	5.99	0.00	18.87	–
11	170	26	1.0	50	66.65	36.72	9.72	12.35	73.24
12	170	94	1.0	50	47.29	36.52	1.55	6.86	84.78
13	170	60	0.16	50	80.14	37.84	17.27	15.68	37.67
14	170	60	1.84	50	50.10	29.95	1.08	10.52	54.58
15	170	60	1.0	8	61.33	35.68	1.35	20.27	63.25
16	170	60	1.0	92	65.21	35.23	4.96	8.23	80.60
17	170	60	1.0	50	58.86	35.50	4.29	10.82	80.86
18	170	60	1.0	50	49.96	33.12	2.50	7.49	87.83
19	170	60	1.0	50	54.25	32.73	3.50	8.96	85.19
20	170	60	1.0	50	50.58	32.41	2.79	7.30	85.66
21	170	60	1.0	50	56.11	32.14	4.66	10.13	87.37
Average of center point(17–21)	170	60	1.0	50	50.58	33.18	3.55	8.94	85.38

All yields are presented on a dry-weight basis.

Nos. 1–8 are the eight factorial points, nos. 9–16 are the eight star points, nos. 17–21 are center points.

The enzymatic digestibility could not be determined under the condition nos.1 and 10 because most sugar solubilized in the spent liquor.

Table 3 Equations for the responses.

Response	Equation[a]	R^2
Residual solid	$Y_{RS}=12.05+2.11T-1.50t-53.25S-0.008T^2+14.62S^2+0.0048E^2$	0.9773
Yield of lignin	$Y_L=192.19-1.49T-1.39t+0.13E+0.005Tt+0.14TS-0.004TE+0.16tS-0.067SE+0.004T^2+4.68S^2+0.002E^2$	0.9749
Yield of glucan	$Y_G=-318.23+4.04T+70.86S-0.45TS-0.33tS-0.01T^2$	0.9739
Yield of xylan	$Y_X=85.02-0.33T-0.84t-34.75S+7.27S^2$	0.9583
Enzymatic digestibility	$Y_{ED}=85.38+21.91T+3.43t+5-3S+5.16E-7.10TS+15.84tE+7.14T^2-2.25t^2-13.88S^2-4.76E^2$	0.9920

[a] T temperature(°C), t time(min), S H_2SO_4 concentration (%), E ethanol concentration (%).

The residual solid and glucan yields decreased below a H_2SO_4 concentration of 1.5% (*w*/*w*). The xylan yield greatly decreased as the H_2SO_4 concentration increased. The solubilization or degradation of the hemicellulosic sugar was very sensitive to the H_2SO_4 concentration. The lignin concentration decreased until a H_2SO_4 concentration of 1.0% (*w*/*w*) and then increased. Lower H_2SO_4 concentrations were insufficient for lignin degradation, while higher H_2SO_4 concentrations caused lignin condensation.

The residual solid yield decreased slightly to a minimum at 50% (*w*/*w*) ethanol and then increased. The effect of the ethanol concentration on glucan was similar to that of the ethanosolv time. As the ethanol concentration increased up to 75% (*w*/*w*), lignin was solubilized or degraded. Above an ethanol concentration of 75% (*w*/*w*), the increment of lignin was small. Delignification occurred because of the result of lignin degradation: the

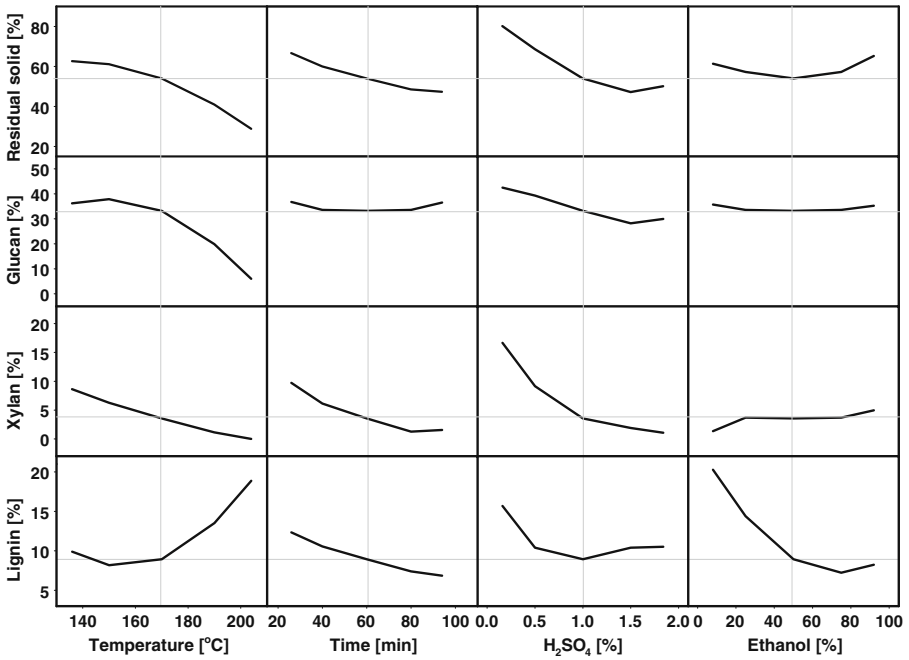

Fig. 1 Effect of the ethanosolv process variables on the yields of residual solid, glucan, xylan, and lignin

lower ethanol concentrations resulted in a higher water chemical activity that promoted acid-catalyzed cleavage of the α- and β-ether linkages in the lignin, whereas the high ethanol concentrations increased solubilization of the lignin [13, 14]. Water in the ethanosolv liquor acted as a decomposing agent for organic material at high temperature and pressure. Ethanol dissolved the hydrophobic component of lignin [15].

Optimum Condition of Enzymatic Digestibility

The small composite design analysis enabled the optimization of the effects of the process variables such as the temperature, time, H_2SO_4 concentration, and ethanol concentration to achieve the maximum enzymatic digestibility (Table 2). The enzymatic digestibility differed as the ethanosolv conditions varied within the experimental range. However, none of the conditions tested produced an enzymatic digestibility that was significantly higher than the center point conditions (85.38%). Further insight into the potential for optimizing the enzymatic digestibility was gained from Fig. 2. Figure 2 shows the response surface plot of the effects of two variables on enzymatic digestibility of ethanosolv-treated barley straw as determined using equation Y_{ED} in Table 3. Ethanosolv times longer than 60 min were useless for enhancing the enzymatic digestibility. The enzymatic digestibility increased to maximum at 1.0% (*w*/*w*) H_2SO_4 and then declined. Additionally, the enzymatic digestibility was maximized 50% (*w*/*w*) for ethanol. Figure 2 indicated that the optimum H_2SO_4 concentration and ethanol concentration conditions existed within the experimental range. However, the enzymatic digestibility increased as the temperature increased. The best condition for the enzymatic digestibility might be at a higher temperature. High temperatures caused excessive degradation, which gave rise to higher hydroxymethyl furfural (HMF) and furfural formations [15–17]. HMF and furfural decrease the ethanol yield and productivity, inhibit growth, or give rise to a longer lag phase. These effects depend on the concentration of HMF and furfural and on the yeast strain used. According to Delgens [16], *Saccharomyces cerevisiae* were inhibited at a HMF concentration of 1.01–3.02 g/l and furfural concentration of 0.96–2.02 g/l. *Zymomonas mobilis* were inhibited at a HMF concentration of 3.02–5.04 g/l and furfural concentration of 0.96–2.02 g/l. HMF and furfural formations in the ethanosolv treatment (170°C, 60 min, 1% (*w*/*w*) H_2SO_4, 50% (*w*/*w*) ethanol) were estimated to be 0.015 and 0.16 g/l; therefore, theses concentration are very lower than that inhibits cell. Therefore, a reasonable temperature was 170°C.

Figure 3 illustrates the enzymatic hydrolysis behavior of the ethanosolv-treated barley straw samples at the center point. The hydrolysis behavior of Avicel and the untreated sample was displayed for comparison. The enzymatic digestibility of the untreated samples was extremely low at a value of 20%. Substantial conversion of the ethanosolv-treated

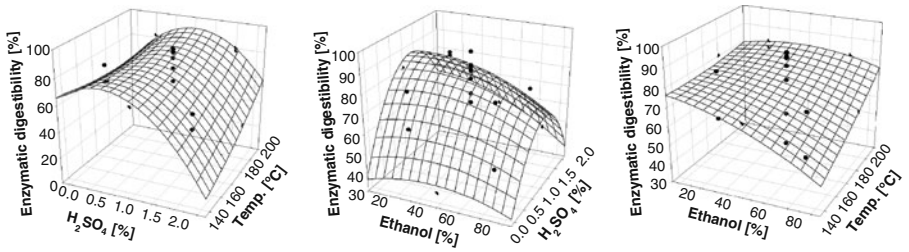

Fig. 2 The surface response plot of the effects of temperature, sulfuric acid concentration, and ethanol concentration on the enzymatic digestibility of ethanosolv-treated barley straw

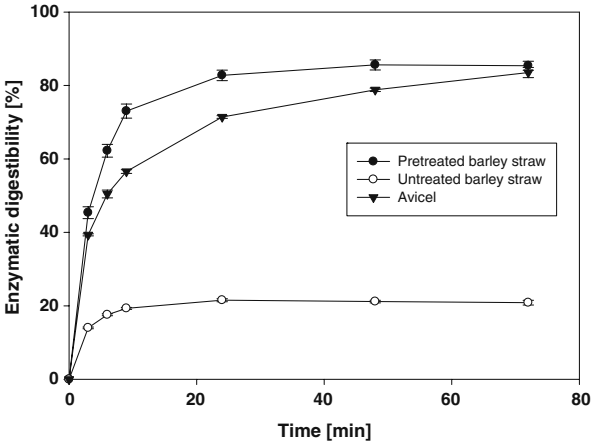

Fig. 3 Enzymatic hydrolysis behavior of the ethanosolv-treated barley straw at the center point conditions (170°C, 60 min, 1.0% (*w*/*w*) H_2SO_4, and 50% (*w*/*w*) ethanol) with enzyme loadings of 20 FPU/g cellulose and 40 CBU/g cellulose

sample was observed within 24 h. When the hydrolysis was extended to 48 h, the conversion of cellulose to glucose reached to 82.8%. The rate of hydrolysis of ethanosolv-treated sample was faster than that of Avicel. The ultimate enzymatic digestibility of the pretreated sample (85.3%) and Avicel were similar (83.5%). Although Avicel was totally made up of cellulose, the hydrolysis behavior of the pretreated samples was superior to Avicel.

Severity Analysis

The effects of the ethanosolv pretreatment conditions on the hemicellulose and lignin content and the enzymatic digestibility were examined. The severity parameter was used to unify the data obtained for different combinations of temperature, time, and catalyst concentration. The severity parameter was calculated as follows [15]:

$$M_0 = tA^n \exp\left[\frac{\mathrm{T} - 100}{14.75}\right] \tag{1}$$

In this equation, t is the reaction time (min), A is the acid concentration (wt.%), n is an arbitrary constant, and T is temperature. Figure 4(a) reveals that the log M_0 was essentially uncorrelated with hemicellulose and lignin content. However, as in Fig. 4(b), the enzymatic digestibility correlated with the log M_0. Therefore, the ethanosolv treatment induced a specific effect on the enzymatic digestibility. From Fig. 4, intensive removal of lignin and hemicellulose was not absolutely necessary for the improved enzymatic digestibility of the barley straw [15].

Mass Balance of Ethanosolv Process

Figure 5 shows the overall mass balance diagram describing the stages from pretreatment to enzymatic hydrolysis. The barley straw was pretreated at the center point (170°C, 60 min, 1.0% (*w*/*w*) H_2SO_4, and 50% (*w*/*w*) ethanol) and a barley straw-to-liquor ratio of 1:7. The

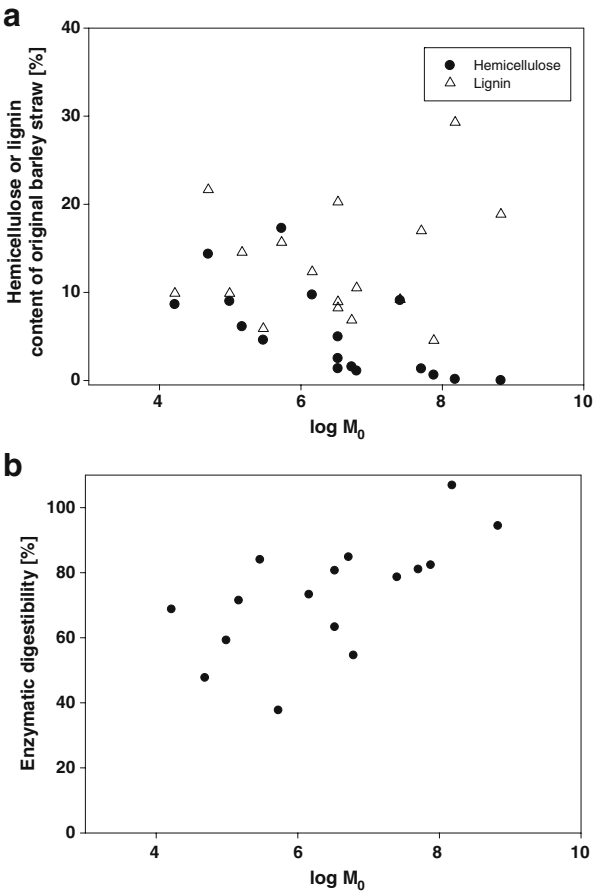

Fig. 4 Effect of the severity parameter on (**a**) the hemicellulose and lignin contents and (**b**) the enzymatic digestibility of the ethanosolv-treated barley straw

pretreated barley straw was then washed with 50% (*w*/*w*) ethanol, which resulted in a 49.42% (*w*/*w*) loss of the solid so that the residual solid was 50.58% (*w*/*w*). Approximately, 85% of the glucan in the untreated barley straw was recovered in the residual solid at the center point conditions. However, substantial losses of xylan were observed with a 14%

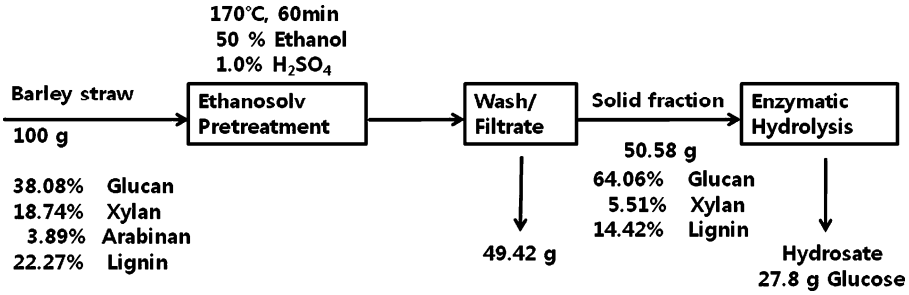

Fig. 5 Mass balance of the overall process at the center point conditions (170°C, 60 min, 1.0% (*w*/*w*) H_2SO_4, and 50% (*w*/*w*) ethanol)

xylan recovery. Such a low xylan recovery indicated that this component was significantly degraded. The residual solid contained 14.42% lignin, compared to 22.27% (*w*/*w*) in the untreated barley straw. Approximately, 32% of the total lignin remained in the residual solid. Next, enzymatic hydrolysis was conducted with enzyme loadings of 20 FPU/g cellulose and 40 CBU/g cellulose. The glucose yield was 27.8 g after 72 h, which was 85.38% of the enzymatic digestibility.

Conclusions

The center point conditions (170°C, 60 min, 1.0% (*w*/*w*) H_2SO_4, and 50% (*w*/*w*) ethanol) for the small composite design were close to the optimal conditions for the enzymatic digestibility. The enzymatic digestibility was the most important parameter for the pretreatment optimization aimed at maximizing the production of ethanol. However, the optimization of the ethanosolv pretreatment in terms of the enzymatic digestibility required a more detailed investigation. The delignification was 68% based on a comparison between the initial weight of lignin before the pretreatment and the weight of lignin in the solid remaining after the pretreatment, and the glucose recovery was 85%. Therefore, the ethanosolv treatment was an effective way to remove lignin and to keep cellulose which was easily converted to ethanol. All of the lignin did not need to be removed to achieve the maximum enzymatic digestibility through severity analysis. In general, the removal of 20–65% of lignin in a sample is believed to be sufficient in order to increase the accessibility of cellulose to the enzymes.

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