Partial Simultaneous Saccharification and Fermentation at High Solids Loadings of Alkaline-pretreated *Miscanthus* for Bioethanol Production

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In this study, alkaline pretreatment at a bench scale (15-L capacity) was performed to obtain a higher solid residue for the SSF (simultaneous saccharification and fermentation) of *Miscanthus sacchariflorus* "Goedae-Uksae 1" (GU) under the following conditions: 1 M NaOH concentration, 150 °C, and 60 min residence time. Compositional analysis and scanning electron microscope analysis revealed the pretreatment to be highly effective for achieving delignification and morphological changes. Spiral impellers were used for the rapid liquefaction of pretreated GU into slurry, and no additional nutrients were added to the fermentation mixture to reduce overall process costs. The SSF was subsequently conducted in a laboratory-scale fermenter (5-L capacity) for 108 to 120 h with 12% and 16% glucan containing pretreated GU. Consequently, 62.8 g/L and 81.1 g/L of ethanol were obtained. Based on these data, the theoretical ethanol yields from 1 kg of GU (dry weight base) were estimated at 164.6 to 171.1 g/L.

Keywords: Alkali pretreatment; Bioethanol; High solids loading; Miscanthus; Simultaneous saccharification and fermentation

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INTRODUCTION

There is a strong interest in bioethanol derived from lignocellulosic biomass because of recent oil crises and global climate change caused by the greenhouse effect (Hill *et al*. 2006). As an additive or substitute for gasoline, bioethanol has great potential because of its complete combustion and lower emissions, and because it can be used without modifying existing car engines (Sørensen *et al*. 2008). Five steps are generally required to produce bioethanol: the pretreatment of biomass, enzymatic hydrolysis, fermentation, distillation, and dehydration (Agbor *et al*. 2011). However, prior to the processes for lignocellulosic bioethanol production, biomass is required as a renewable energy source (Erb *et al*. 2012). Biomass, such as agricultural residues and bioenergy corps, generally requires cultivation, collection, transportation, and storage for bioethanol production (Meehan *et al*. 2013).

Typical *Miscanthus* species consist of 40 to 60% cellulose, 20 to 40% hemicellulose, and 10 to 30% lignin (Brosse *et al*. 2012). Cellulose (or hemicellulose) is a major source of fermentable sugar for bioethanol production. However, cellulose binds with hemicellulose and lignin in fibrous plants, which contributes to its recalcitrance

(Sánchez and Cardona 2008). In addition, cellulose has a highly crystalline structure that is insoluble in water and resistant to depolymerization (Zheng *et al*. 2009). The pretreatment of lignocellulosic biomass is therefore necessary to disrupt the tight structure of cellulose, hemicellulose, and lignin, facilitating the conversion of biomass into fermentable sugars (Hendriks and Zeeman 2009; Alvira *et al*. 2010). Pretreatment includes physical, chemical, and biological processing, depending on the type of lignocellulosic biomass and catalyst used (Mosier *et al*. 2005).

Each method has advantages and disadvantages (Brodeur *et al*. 2011). For instance, pretreatment with weak acid is known to be a comparatively simple and conventional method, but it produces fermentation inhibitors, such as furfural and 5-HMF (Taherzadeh and Karimi 2008). Alkaline pretreatment using NaOH significantly removes lignin, but produces large quantities of process wastes (liquid fraction) (Chen *et al*. 2013). Pretreatment is widely regarded as one of the most expensive processes and strongly influences downstream processes for bioethanol production, such as enzymatic hydrolysis and fermentation (Kumar *et al*. 2009). Thus, many pretreatment techniques have been considered for low-cost approaches and for generating high sugar yields (Kumar and Murthy 2011).

In addition to pretreatment, sugar and ethanol yields have significant effects on processing costs (Yang and Wyman 2008). To be economically viable, a minimum ethanol concentration of 39.1 g/L (approximately 5% ethanol) must be produced in industrial-scale distillation operations (Varga *et al*. 2004). Therefore, higher sugar concentrations should be achieved by increasing the solids loading in the reactor, which leads to increased ethanol production (Olofsson *et al*. 2008). Several studies have shown the potential for the industrial application of high solids loadings of pretreated biomass (Kristensen *et al*. 2009; Li *et al*. 2013). However, increasing concentrations of substrates can lead to decreased ethanol yield due to inefficient mixing and mass transfer in fermentation reactions (Modenbach and Nokes 2012; 2013).

In this study, alkaline pretreatment was performed with *Miscanthus sacchariflorus* "Goedae-Uksae 1" (GU). Giant *Miscanthus*, or "Goedae-Uksae" in Korean, can reach 4 m in height, 9.6 mm in stem thickness, and has dimensions two-fold higher than those of common *Miscanthus* species (Moon *et al*. 2010). GU is cultivated on "Youngan" and "Ungpo" in the Kum river area of 148 ha in Korea as an energy crop because of its high yield potential (up to approximately 30 t/ha).

Herbaceous crops and agricultural residues are reported to be suitable for alkaline pretreatment (Brosse *et al*. 2012). The major advantage of alkaline pretreatment is delignification, which enhances enzymatic accessibility to cellulose and, therefore, saccharification (Park and Kim 2012). It has been reported that the cellulose recovery of *Miscanthus sinensis* following pretreatment with soda was the highest among different reagents (Serrano *et al*. 2010).

Pretreatment was performed at the bench scale (15-L capacity) with fabricated equipment for bioethanol production from GU. After the pretreatment of GU, nutrient (or buffer) requirements and impeller type were determined for efficient simultaneous saccharification and fermentation (SSF). Finally, fermentation of pretreated GU was performed at 593 g/L and 790 g/L loadings, corresponding to 12% and 16% glucan concentration (dry weight base), respectively, in a 5-L fermenter, then overall processes were analyzed.

EXPERIMENTAL

Materials

GU was harvested from Muan, Korea in 2013. After cutting, milling, and sieving, GU samples of less than 3 mm were obtained (Korea Pulverization Machinery Co., Inchon, Korea). They were dried in an oven at 60 °C for 24 h, then stored in a desiccator.

Methods

Alkaline pretreatment

A solid-to-liquid ratio of 1:9 GU and 1.0 M NaOH were mixed in a 15-L reactor equipped with pressure and temperature sensors. The reactor was set to 150 °C for 60 min, and the mixture was agitated at 60 rpm during the pretreatment reaction. The jacket surrounding the reactor was filled with heated oil, which was the primary heat source. After the reaction finished, pressurized N_2 gas was loaded into the vessel to 10 bars to collect pretreated samples into a cyclone separator. The pretreated GU was washed with tap water for neutralization, filtered through a non-woven fabric bag, and then used for enzymatic saccharification or fermentation.

Scanning electron microscopy (SEM) analysis

A scanning electron microscope (TM-100, Hitachi; Tokyo, Japan) was used to analyze changes in the physical structure of GU before and after alkaline pretreatment. The SEM was operated at an accelerating voltage of 15 kV under a vacuum.

Partial SSF of pretreated GU at high solids concentrations

Partial SSF was conducted according to the procedure described in NREL/TP-510-42630 (Dowe and McMillan 2001) and NREL/TP-510-42629 (Selig *et al*. 2008), with minor modifications. The reaction was carried out in a 5-L fermenter with an agitation speed of 150 rpm for 108 to 120 h. The reaction slurry consisted of 593 g/L and 790 g/L loadings of pretreated GU (wet-weight), 30 filter paper units (FPU)/g cellulase (Novozymes, Cellic CTec2), and sterile water. Exponentially-grown *Saccharomyces cerevisiae* CHY1011 (Han *et al*. 2011) in 50 mLYPD medium (10 g of peptone, 5 g of yeast extract, and 20 g of glucose in 1 L) were harvested by centrifugation at 15,000 rpm for 10 min at 4 °C. Harvested cells were washed with 10 mL of sterilized water and added to the fermentation mixture, resulting in a 1 L fermentation working volume. The initial cell density measured by colony counting was estimated at approximately 8.4×10^6 CFU/mL. Before adding yeast cells to the reaction mixture, enzymatic hydrolysis was conducted for an initial 24 h at 50 °C. Then, SSF was performed at 33 °C for an additional 84 to 96 h with an agitation of 150 rpm after the addition of yeast cells. Samples were taken periodically to determine ethanol and sugar concentrations using gas chromatography (GC) and high-performance liquid chromatography (HPLC), respectively. Before loading onto the analytical columns, samples were centrifuged at 13,000 rpm for 10 min, filtered through a 0.22-μm membrane, and boiled for 10 min to deactivate the enzyme, if necessary. The HPLC (Waters, Milford, MS, USA) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) was set to 65 °C. The mobile phase was 0.5 mM H_2SO_4, delivered at a constant flow rate of 0.6 mL/min. Peaks were detected using a refractive index detector (Waters, 2410; USA) and identified by retention time. Quantification was performed using a calibration curve generated for each sugar. An HP-Innowax 19091N-133 column (Agilent, USA) for GC was used to capture ethanol with helium gas (15 mL/min) as the

carrier, and ethanol was detected by a flame ionization detector (FID, Agilent, USA) at 210 °C.

Chemical analysis

Compositional analysis of GU was performed according to NREL/TP-510-42618 (Sluiter *et al*. 2008). After a two-step acidic hydrolysis, sugar concentration in the hydrolysis liquid was determined by HPLC. Ash content was determined according to NREL/TP-510-42622 (Sluiter *et al*. 2005). Moisture content was determined using an HR83 halogen moisture analyzer (Mettler-Toledo, Schwerzerbach, Switzerland). For chemical composition analysis, such as contents of nitrogen and phosphate, GU samples were analyzed by the Foundation of Agri. Tech. Commercialization & Transfer, Korea.

RESULTS AND DISCUSSION

Alkaline Pretreatment of GU and Compositional Changes Before and After Pretreatment

The GU was incubated with 1 M NaOH (w/v) at a solid-to-liquid ratio of 1:9 at 150 °C for 60 min. The particle size of GU was determined according to previous studies, showing a higher saccharification rate with less than 3 mm of biomass (Wang *et al*. 2010; Kang *et al*. 2012; Haque *et al*. 2013). The pretreatment conditions compared with the previous studies are summarized in Table 1. Generally, pretreatment is influenced by NaOH concentration, liquid-to-solid ratio, temperature, and residence time (Hendriks and Zeeman 2009). Alkaline pretreatment in most studies was carried out in an autoclave or oil bath at 100 to 145 °C with 1 to 7% NaOH. Overall, increased temperature conditions were accompanied by decreased NaOH concentrations and residence times. The main difference between this study and the previous NaOH pretreatment was an explosion caused by pressured nitrogen gas. However, this explosion was necessary to easily collect the samples from the reactor due to its structural limitation through the cyclone system. Choi *et al*. (2013) optimized NaOH-catalyzed explosion pretreatment using response surface methodology (RSM) with the following conditions: 3% NaOH, 160 °C, and 11.3 min. The pressurized nitrogen gas was 20 bars (Choi *et al*. 2013), while that of the nitrogen in this study was 10 bars. The shorter residence time and lower NaOH in comparison with this study was because of an additional soaking step at room-temperature for 12 h with 3% NaOH (Choi et al. 2013). However, direct comparisons might be difficult because: (1) different pretreatment conditions (as shown in Table 1) reflect that the effectiveness of NaOH is strongly dependent on the chemical and physical characteristics of biomass feedstock; (2) the biomass-to-NaOH ratio varies among studies; and (3) particle size varies. Thus, pretreatment efficiency is determined by downstream processes such as enzymatic hydrolysis and fermentation (Kumar *et al.* 2009).

The compositional analysis of raw biomass indicates that GU was composed of 40.3 ± 1.4 wt% cellulose, 23.6 ± 0.4 wt% hemicellulose, 24.4 ± 0.3 wt% lignin, and 3.0 ± 0.1 wt% ash. After pretreatment, 40.2 ± 0.2% solids (dry weight base) was recovered and the solids consisted of 80.9 ± 1.0 wt% cellulose, 12.3 ± 1.8 wt% hemicellulose, 5.0 ± 0.2 wt% lignin, and 1.0 ± 0.1 wt% ash. Cellulose, the major fermentable sugar source, was recovered up to 80.3%, while up to 92% of initial lignin content was removed.

Table 1. Studies of Sodium Hydroxide Pretreatment with a Variety of Conditions and Biomass

Biomass	Conditions					Saccharification and (or) Fermentation			Ethanol Yield[b] (%)	References
	Particle Size (mm)	S:L[†]	NaOH (%, w/w)	Temp (°C)	Residence Time (min)	Dosage (Enzyme g/g Cellulose)	Time (h)	Efficiency[a] (%)		
Barley	20.00 to 25.00	1:9	2.00	105.0	10.0	20 FPU (cellulase) 40 IU (ß-glucosidase)	72	86.5	·	Haque *et al*. 2012
Birch Spruce	0.80	NS[†]	7.00	100.0	120.0	20 FPU (Celluclast 1.5 L) 50 IU (Novozyme 188)	96	82.3 35.7	54.7[c] 26.0[c]	Mirahmadi *et al*. 2010
Coastal Bermuda grass	2.00	1:10	0.75	121.0	15.0	40 FPU (cellulase) 70 CBU (cellobiase)	72	90.4	·	Wang *et al*. 2010
Cogon grass	20.00	1:20	10.00	15.0 to 20.0	1440.0	0.255 mL/g WIS[1] (Accellerase 1000)	72	·	76.2[d]	Lin and Lee 2011
Empty fruit bunch	5.00	1:5/ 20	3.00	160.0	11.3	40 FPU[*] (CTec 2)	72	88.8	88.0[d]	Choi *et al*. 2013
Elephant grass	3.00	1:20	2.00	120.0	60.0	30 FPU (Accellerase 1500)	26	82.0	95.0[d]	Cardona *et al*. 2014
Wheat straw	40.00 to 60.00	1:20	1.00	121.0	60.0	35 FPU (NS)	120	·	70.7[d]	Zhang *et al*. 2013
Switch grass	2.00	1:10	1.00	50.0	720.0	15 FPU (cellulase) 20 CBU[**] (cellobiase)	72	74.4	·	Xu *et al*. 2010
Switch grass	2.00	1:10	1.00	121.0	30.0	40% (CTec 2) and 6% (HTec 3) g/g dry biomass	72	78.7	·	Wang *et al*. 2012b
Poplar	2.00	1:8	2.80	94.0	60.0	15 FPU (cellulase)	48	42.2	·	Rawat *et al*. 2013
Rapeseed straw	0.71 to 1.40	1:10	7.90	68.4	330.0	30 FPU (cellulase) 30 IU (ß-glucosidase)	·	94.0	·	Kang *et al*. 2012
Miscanthus	1.00	1:9	2.50	105.0	10.0	15 FPU (cellulase) 30 IU[***] (ß-glucosidase)	72	87.0	·	Haque *et al*. 2013

Table 1. cont.

Miscanthus	1.00 to 3.00	1:6	5.90	145.3	29.0	50 FPU (cellulase) 30 CBU (ß-glucosidase)	72	90.0	84.6[c]	Han *et al*. 2011
Miscanthus	3.00	1:9	4.00	150.0	60.0	30 FPU (Ctec 2)	96	·	91.4[e]	This study

[*]FPU = Filter paper Unit; [**]CBU = Cellobiase Unit; [**]IU = International Unit
[1]WIS = Water-Insoluble-Solids
[†]S:L = Solid-to-liquid ratio·
[††]NS = not specified
[a]Efficiency (%)=reducing glucose (g/L)x0.9x100/initial glucose (g/L)
[b]Ethanol yield (%)=produced ethanol (g/L)/initial glucose (g/L)x0.512x100
[c]SHF = separate hydrolysis and fermentation
[d]SSF = simultaneous saccharification and fermentation
[e]pSSF = partial simultaneous saccharification and fermentation

The substantial reduction of lignin content is an important concern because the degree of delignification could determine the efficiency of alkaline pretreatment for further saccharification (Chang and Holtzapple 2000; Li *et al*. 2013). It could be argued that the loss of hemicellulose was approximately 79%. This could be the reason that pretreatment conditions were comparably more severe than the conditions of previous reports (Table 1).

However, only 6-carbon fermentable yeast was used in this study. Further study will be focused on the optimization of pretreatment methods to increase hemicellulose content together with microbial strains development for 5-carbon utilization (*e.g.*, xylose).

Structural Changes of GU Before and After Pretreatment

Scanning electron microscopy images of GU before and after pretreatment are shown in Fig. 1. Untreated GU samples showed tight, intact surfaces consisting of hemicelluloses, lignin, and binding materials (Fig. 1a), while pretreated samples were cracked, scattered, and developed heterogeneous structures throughout the biomass (Fig. 1b). Cellulose fibers were distinctly opened from the complex of the homologous bundles after pretreatment (Fig. 1b). These reflect effective delignification by NaOH, as similar observations have been reported in cogon grass and *Miscanthus sinensis* (Lin and Lee 2011; Haque *et al*. 2013). It is expected that the increased pore size and surface area in pretreated GU could contribute to enhanced enzyme accessibility and hydrolysis.

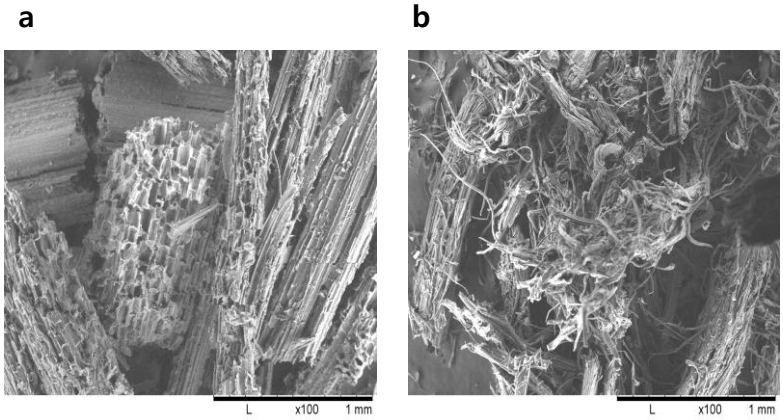

Fig. 1. Scanning electron microscopy (SEM) analysis. Micrographs from untreated (a) and treated (b) *Miscanthus* (x100)

Partial SSF with Pretreated GU at High Solids Loadings

Additives (*e.g.*, nutrients) required for fermentation could play an important role in the overall cost reduction of bioethanol production (Kadam and Newman 1997). For example, dry distiller's grain and soluble, major byproducts of corn-based ethanol production, were used as external nutrient supplements in SSF with high solids loadings of pretreated corn stover (Lau *et al*. 2008). Here, to estimate whether YP (yeast extract and peptone) or a citrate buffer influenced on yeast fermentation, four different medium conditions were prepared depending on with/without YP and the buffer. Figure 2 shows kinetics of ethanol production in a 250-mL flask in SSF at four different medium conditions. For the purpose of evaluation of ethanol production glucose consumption rates were eliminated. As expected, yeast cultivated with YP and the buffer exhibited the highest ethanol productivity (g/L/h) and yield (0.87 g/L/h and 92.1%, respectively), due to stable enzyme reaction by the buffer, resulting in highest glucose conversion rate; and 2)

sufficient nutrients provided by YP. The final ethanol concentration in the medium containing all supplements was approximately 62 g/L, while that in the medium with no supplement was approximately 56 g/L. Although the ethanol concentration in the reaction mixture without supplements was clearly lower, the theoretical ethanol yield of the mixture without additives was more than 82%. Considering the potential cost reduction (weighing lower ethanol concentrations against supplementation), it was decided that SSF be performed without the use of additives, because the yeast can grow and ferment converted glucose from GU into ethanol. It was reported that raw *Miscanthus* contains 1.7% protein (Vanderghem *et al*. 2012), 0.6% potassium, 0.1% chloride (Jørgensen 1997), and 0.2% phosphate.

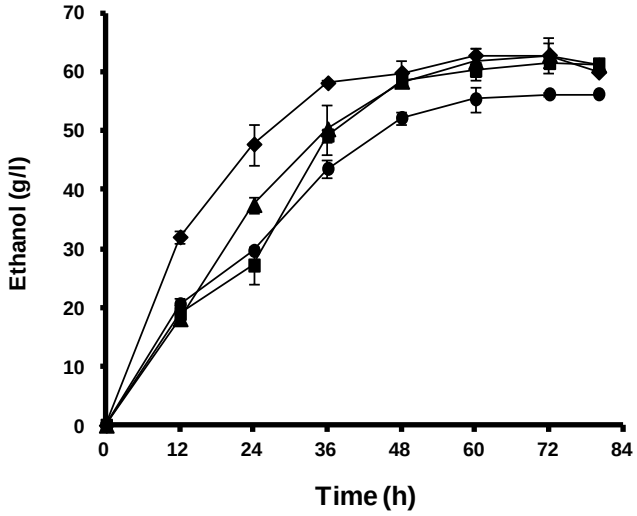

Fig. 2. Determination of additional nutrients for the SSF of pretreated GU. The fermentation mixture with YP and buffer (◆); with YP and without buffer (▲); without YP and with buffer (■); and without YP and buffer (●). Bars represent standard deviation from two independent experiments

Enzymatic saccharification or fermentation with high solids generally induces a lack of available water, high viscosity, and thereby an insufficient transfer of biomass and heat in reaction. Therefore, the rapid liquefaction of pretreated biomass is a key factor in determining fermentation productivity and yield (Jørgensen *et al*. 2007). Thus, the impeller was characterized according to mixing behavior; the use of the plate-and-frame, double-curved-blade impeller, and peg mixer was at first avoided because of limited power input and structure in the 5-L fermenter (Modenbach and Nokes 2012). Instead, a Spiral impeller was equipped in the fermenter, as reported previously (Zhang *et al*. 2010). The maximum solid loading in pretreated GU was determined to be 16% glucan (790 g/L of solid, wet weight base), because solids containing more than 16% glucan prevented the impeller from proper mixing because of high viscosity.

The efficiency of the alkaline pretreatment of GU was evaluated by partial SSF. The collected pretreated GU containing 75 wt% of moisture contents was 1.6 kg from 1 kg raw materials, and its cellulose contents was 80.9 wt%. Thus, a glucan loading of 12 % to 16 % (w/v) in a 1-L working volume was achieved by loading 14.8 wt% and 19.7 wt% of pretreated GU solids (dry weight base). This solids concentration was reached stepwise over 4 h accompanied by the addition of enzymes and water to avoid the rapid generation

of high viscosity. Such fed-batch schemes have been investigated for saccharification or fermentation with high solids loadings (up to 25%, based on dry weight) (Hodge *et al*. 2009; Wang *et al*. 2012a; Zhang *et al*. 2013). In addition, the concept of increasing solids concentrations in a stepwise manner is a continuous process in which pretreated biomass accumulates gradually in a saccharification or fermentation tank (Schell *et al*. 2003; Han *et al*. 2013). Figure 3 shows the kinetics of partial SSF with 12% and 16% glucan-containing pretreated GU. Converted glucose concentrations increased rapidly up to 109 g/L (Fig. 3b) during the saccharification phase for the first 24 h, which indicates that the stirring system equipped with a spiral impeller was effective for biomass mixing and diffusion (supplementary Fig. 1). After 48 h, following the addition of yeast at 24 h, the fermentation rate seemed to keep up with the saccharification rate because the glucose concentration was consistently less than 5 g/L. Maximum ethanol concentrations for 12% and 16% glucan loadings were 62.8 ± 2.0 g/L (Fig. 3a) and 81.1 ± 2.0 g/L (Fig. 3b), corresponding to theoretical ethanol yields of 93.0 % and 90.9 %, respectively. This phenomenon appeared in many other studies, due to inefficient mixing and mass transfer with increased solids concentration (Jørgensen *et al.* 2007; Han *et al.* 2011).

Meanwhile, non-utilized xylose concentration was the same as 10.5 g/L and 13.0 g/L from 12% and 16% glucan, respectively, containing GU. Because Cellic Ctec II (Novozymes) mainly consists of cellulase, xylose concentration would be almost the same as average 11.7 g/L in both 12% and 16% glucan-containing pretreated GU.

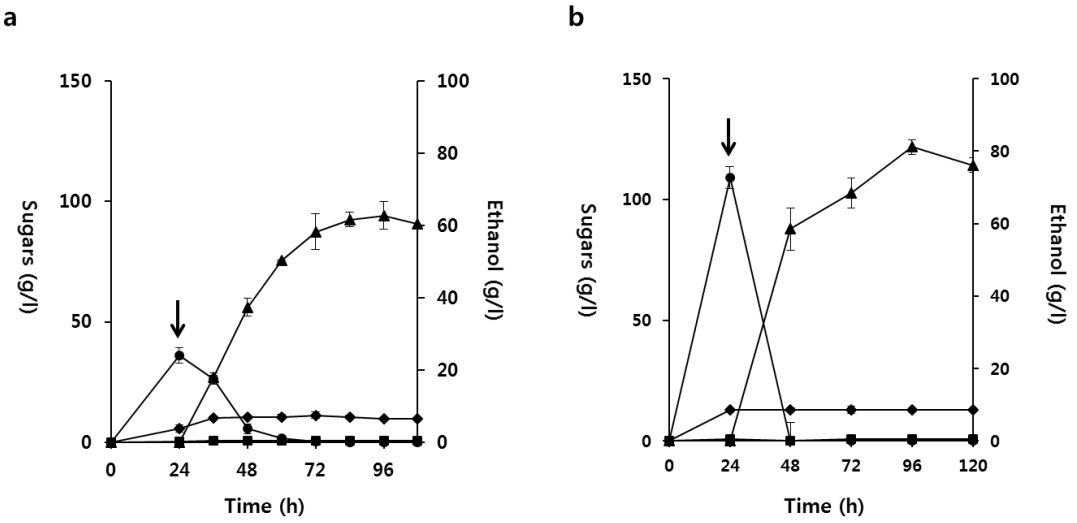

Fig. 3. Partial SSF kinetics with pretreated GU containing 12% glucan (a) and 16% glucan (b); ethanol (▲), glucose (●), xylose (◆), and arabinose (■). Fermentation was conducted in a 5-L reactor. Arrows indicate cell addition at 24 h. Bars represent standard deviation from three independent experiments

Overall Process

The overall process following pretreatment and fermentation is shown in Fig. 4. Under the described pretreatment conditions, approximately 80.6% cellulose was recovered, and 92% lignin was removed. In these respects, the alkaline pretreatment conditions of 1 M NaOH, 150 °C, and 60 min at the bench scale (15-L capacity) were highly effective. High rates of lignin removal were the reason only 42% solids residue was obtained from 1 kg of GU (dry base weight) after pretreatment. For partial SSF for 72 h,

approximately 90% of the theoretical ethanol yield was achieved from up to 20% (w/v) solids (dry weight basis, glucan 16%) without the addition of extra nutrients, such as peptone and yeast extract. Therefore, theoretical ethanol was estimated as 171.7 g/L from 1 kg GU biomass, based on the high solids fermentation.

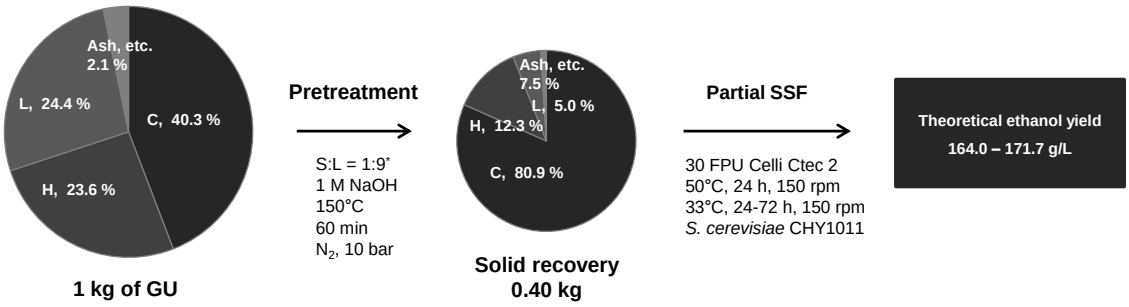

Fig. 4. Overall processes based on dry matter content. C = cellulose; H = hemicellulose; and L = lignin. *S:L = solid:liquid ratio

CONCLUSIONS

1. Bench-scale pretreatment (15-L capacity) under the conditions of 1 M NaOH, 150 °C, and 60 min was demonstrated to be effective, resulting in a significant increase of cellulose content from 40% to 80%, along with significant lignin removal (up to 91%). However, unintended hemicellulose degradation caused by pretreatment should be overcome with further study. The effects of varied pretreatment factors, such as alkali concentration, reaction temperature, and reaction time should be investigated.
2. Yeast can grow and ferment in pretreated *Miscanthus sacchariflorus* "Goedae-Uksae 1" (GU) slurry without any nutrient supplementation when GU solids are enzymatically hydrolyzed. Thus, pretreated GU has the potential for reducing fermentation costs. Liquid fraction (LF) obtained during the pretreatment has the phenolic derivatives from lignin and shows high pH (Minu *et al.* 2012). It is surely hazardous to aquatic organisms. Therefore, LF could be recycled as a pretreatment solution and lignin can be extracted in further study. In addition, the water consumption for neutralization of pretreated GU should be minimized to reduce overall costs. Alternatively, LF and wasted water might be combined and tested for further pretreatment following the adjustment of NaOH concentration. Enzyme dosage also would be varied in high solids fermentation, because the enzyme costs account for 25% of total process in ethanol production (Brodeur *et al.* 2011).
3. Partial SSF with a 19% (w/v) solids loading (16% glucan concentration) yielded 81.1 ± 2.0 g/L of bioethanol, corresponding to a 90.9% theoretical yield. Therefore, the present study is a significant contribution to bioethanol production from *Miscanthus*.

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APPENDIX

Supplementary Fig. 1. Two Impellers used in this study for high solids fermentation and performance of mixing behaviors by the impellers.