Research Article

Evaluation of Alkali-Pretreated Soybean Straw for Lignocellulosic Bioethanol Production

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Soybean straw is a renewable resource in agricultural residues that can be used for lignocellulosic bioethanol production. To enhance enzymatic digestibility and fermentability, the biomass was prepared with an alkali-thermal pretreatment (sodium hydroxide, 121°C, 60 min). The delignification yield was 34.1~53%, in proportion to the amount of sodium hydroxide, from 0.5 to 3.0 M. The lignin and hemicellulose contents of the pretreated biomass were reduced by the pretreatment process, whereas the proportion of cellulose was increased. Under optimal condition, the pretreated biomass consisted of $74.0 \pm 0.1\%$ cellulose, $10.3 \pm 0.1\%$ hemicellulose, and $10.1 \pm 0.6\%$ lignin. During enzymatic saccharification using Cellic® CTec2 cellulase, 10% (w/v) of pretreated soybean straw was hydrolyzed completely and converted to 67.3 ± 2.1 g/L glucose and 9.4 ± 0.5 g/L xylose with a 90.9% yield efficiency. Simultaneous saccharification and fermentation of the pretreated biomass by *Saccharomyces cerevisiae* W303-1A produced 30.5 ± 1.2 g/L ethanol in 0.5 L fermented medium containing 10% (w/v) pretreated biomass after 72 h. The ethanol productivity was 0.305 g ethanol/g dry biomass and 0.45 g ethanol/g glucose after fermentation, with a low concentration of organic acid metabolites. Also, 82% of fermentable sugar was used by the yeast for ethanol fermentation. These results show that the combination of alkaline pretreatment and biomass hydrolysate is useful for enhancing bioethanol productivity using delignified soybean straw.

1. Introduction

The soybean (*Glycine max* L. Merr.) is an annual herbaceous plant in the species of legumes that originated in East Asia (including China, Japan, and Korea), widely grown for its edible bean as an important food resource. During soybean harvesting, the stalks, the husks, and the dry leaves remain as the agricultural residues. In Korean traditional agriculture farming, the dry leaves had been traditionally used as cattle feedstock, but the rest of residues had been consumed as biomass fuel for heating. However, the modern agriculture farm does not use these agricultural residues anymore and abandon the large volumes of the biomass [1].

Nevertheless, soybean straw, the residual part, has the potential to serve an inexpensive feedstock for the production of fermentable sugars, instead of food sources, such as corn, sugar cane, and other food stocks, for the production of bioethanol or other biorefinery products [1, 2]. Among various biomass sources, crop residues such as rice, wheat, barley straw, and corn stover have gained considerable interest and several studies have already been reported based on these feedstocks [3–5]. However, soybean straw, like other lignocellulosic biomaterials, consists of a rigid cellulose structure of strongly cross-linked amorphous hemicellulose and lignin [6–10]. These rigid biomass structures are too chemically complex and too resistant to enzymatic hydrolysis for the production of fermentable sugars. Thus, pretreatment is necessary to change the rigid lignocellulose structures into more enzymatically accessible and digestible forms [2–5].

Soybean straw contains a relatively low level of hemicellulose and lignin per gram biomass, compared with other lignocellulosic biomasses [6–10]. Thus, pretreatment is needed to increase the cellulose content and to decrease the hemicellulose and lignin contents in the biomass. The pretreatment processes should enhance the proportion of cellulose in soybean straw [7–10]. Acidic, alkali, and sequential acidic-alkali pretreatments, combined with high temperature or high pressure, have been applied in "conventional" chemical

treatment processes [3–5, 11–14]. Acidic pretreatments are known to be effective with lignocellulosic biomasses in reducing the hemicellulose content. Similarly, alkali pretreatments have been reported as simple processes for the delignification of biomass under mild conditions with minimal sugar degradation and without the formation of inhibitory compounds [14]. Thus, chemical pretreatments can reduce hemicellulose and lignin together to enhance the cellulose content of a biomass [11, 13, 14].

In this study, an alkali-pretreatment was carried out to decrease hemicellulose and lignin for high cellulose content in soybean straw. The biomass was pretreated under different concentration of sodium hydroxide at high temperature, that is, 121°C. The effectiveness of the alkali-pretreatment of the biomass was determined based on the degree of compositional change and enzymatic hydrolysis of pretreated solids. In addition, to evaluate the alkali-pretreatment process, the simultaneous saccharification and fermentation was also performed using an ethanologenic yeast strain of *Saccharomyces cerevisiae* and ethanol productivity was determined.

2. Materials and Methods

2.1. Materials. Soybean straws were obtained locally from a farm in Jeongeup, Jeonbuk, South Korea, at the end of November 2017. It was washed with water to eliminate soil and other particles and then dried at 105°C for 24 h. The straws were chopped into 5–10 cm lengths for chemical pretreatment. Cellic CTec2 cellulase was provided by Novozymes Korea (Seoul, Republic of Korea).

2.2. Chemical Pretreatment of Soybean Straws. Dried straws (20% w/v) with no physical treatment were soaked in sodium hydroxide (NaOH) solution in the concentration range of 0~3 M and heated in an autoclave (121°C, 15 psi, 60 min). The thermal-alkali-pretreated biomasses were removed from the black alkali solution and then washed with flowing tap water to remove NaOH from the biomass. This washing step was repeated several times until the washed water showed a pale brown color. The alkali-pretreated straws were dried at 105°C for 24 h to reduce the moisture content and then stored under anhydrous conditions. The composition of the pretreated biomass was analyzed based on the NREL chemical analysis and testing laboratory analytical procedures (LAPs) of the US Department of Energy (DOE). The lignin content of the biomass was analyzed according to the LAPs of the DOE (LAP-003 and LAP-004). The biomass pretreatment with different NaOH concentration was performed in triplicate.

2.3. Enzymatic Hydrolysis of the Alkali-Pretreated Soybean Straw. Enzymatic hydrolysis of the alkali-pretreated straw (2 M NOH-treated biomass) was carried out in a tube with a 30 ml reaction volume using Cellic CTec2 cellulase. 10% (w/v) pretreated biomass was soaked in phosphate buffer (pH 6.0) containing the cellulase with 10~50 FPU (filter paper unit) per gram of dry biomass. Enzymatic hydrolysis was performed at 42°C, 200 rpm, for 48 h. The biomass hydrolysate was withdrawn at each 12 h for 72 h. After centrifugation of the

enzyme reactants, the hydrolyzed products were analyzed by high-performance liquid chromatography (HPLC) for the amount of monosaccharides generated in the enzymatic hydrolysis. The enzyme solution of Cellic CTec2 cellulase used for the enzymatic hydrolysis basically contains 206 ± 2.3 g/L glucose and 193.3 ± 0.2 g/L xylose. The calculation of the amount of monosaccharides (glucose and xylose) hydrolyzed from the pretreated biomass and the enzymatic saccharification yields excludes the amount of these sugars from the solution of the cellulase.

2.4. Ethanol Production Strain, Growth Conditions, and Fermentation. *Saccharomyces cerevisiae* W303-1A was used as an ethanol production strain [12]. The ethanologenic strain was cultivated in YPD broth (2% Bacto peptone, 1% Bacto yeast extract, and 2% glucose) at 30°C at 200 rpm for 24 h. To prepare a seed culture for ethanol fermentation, 1% (v/v) seed culture was inoculated in a medium containing enzymatic hydrolysis solution, in which 10% (w/v) pretreated soybean straw was hydrolyzed by the cellulase for 48 h as carbon sources, 2% Bacto peptone, and 1% Bacto yeast extract as nutrient sources. Then the seed culture was further cultivated at 30°C at 200 rpm for 24 h. 5% (v/v) preculture of the yeast strain was inoculated into a 1-L fermentor FMT ST-S (Fermentec, Cheongju, South Korea) with a 0.5 L working volume containing 10% (w/v) alkali-pretreated soybean straw, 2% Bacto peptone, and 1% Bacto yeast extract and then the fermentor was operated at 30°C with agitation at 300 rpm. The ethanol fermentation was performed in triplicate.

2.5. Analytical Procedures. Cell growth was monitored by measuring the optical density at 600 nm (OD600 nm) using a spectrophotometer. Total reducing sugars in the enzymatic saccharification reaction were measured using the 3,5-dinitrosalicylic acid method. The amounts of the released sugars in the enzymatic saccharification and the metabolites in fermentation were determined with a high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA), equipped with a refractive index detector, an autosampler, and an Aminex HPX-87P column (7.8 × 300 mm; BioRad, Hercules, CA, USA) for monosaccharide analysis or a Rezex ROA-Organic Acid H^+ column (7.8 × 300 mm; Phenomenex, Torrance, CA, USA) for organic acid analysis. All samples were clarified by filtration with a 0.20-μm filter (Acrodisc LC PVDF Minispike; Pall Life Sciences, Ann Arbor, MI, USA) and then injected into the analytical HPLC column. The column temperature was kept at 65°C. The mobile phase was distilled water for monosaccharides and 2.5 mM sulfuric acid for organic acids, with a flow rate 0.5 mL/min under isocratic conditions. Under these conditions, cellobiose, glucose, xylose, ethanol, glycerol, and xylitol were detected at the retention times 9.93, 12.08, 13.14, 16.36, 18.96, and 34.33 min, respectively, in monosaccharide analyses. Succinic acid, lactic acid, acetic acid, and ethanol were detected at the retention times 13.14, 16.36, 18.96, and 34.33 min, respectively, in organic acid analyses. All analyses were performed in triplicate

TABLE 1: The chemical composition of soybean straws.

Sample	Compositions (%) per 100 g soybean straw*					
	1	2	3	4	5	6
Cellulose	44–83	25.0	34.1	39.8	35.3	44.2
Hemicellulose	-	11.9	16.1	22.6	16.9	5.9
Lignin[2]	5–14	17.6	21.6[1]	12.8	21.7[1]	19.2[1]
Ash	2–5	-	5.2	-	10.6	-
Reference	[6]	[7]	[8]	[9]	[10]	This study

*The percentage of composition in the biomass is based on the dry weight of soybean straw. [1]The amount of lignin contains acid-soluble and insoluble materials. [2]The lignin means acid-insoluble fraction.

3. Results and Discussion

3.1. Alkali Treatment of Soybean Straw and Its Composition. Prior to alkali-pretreatment, cellulose, hemicellulose, and lignin contents in the soy straw raw material were determined by using the methods of the US DOE. Dried soybean straw (100 g) consisted of 44.2% cellulose, 5.9% hemicellulose, and 19.2% lignin (Table 1). Compared to these contents in several different biomasses summarized in Table 1 [6–10], the soybean straw used in this study contained a relatively low amount of hemicellulose and a high content of lignin. It needs to reduce lignin content to enhance the enzymatic accessibility and digestibility to produce a fermentable sugar glucose and to enhance ethanol production yield using an ethanologenic strain [5].

To enhance a high-cellulose-content biomass, alkali-pretreatment was applied to reduce lignin in the soybean straw (Figure 1). After the biomass had dried completely, the chemical composition change in alkali-pretreated soybean straws was analyzed (Table 2). The concentration of sodium hydroxide affected the solubility of the biomass and the loss of cellulose, hemicellulose, and lignin contents. Alkali-thermal treatment within sodium hydroxide extracted up to 52~64.5% of the biomass into the soluble fraction, even though hot water without any alkali compound extracted 29.1% of the biomass. With 3 M sodium hydroxide pre-treatment, the insoluble residue fraction contained 72.9% cellulose, 9.1% hemicellulose, and 9.0% lignin per 100 g dry biomass. After alkali treatment, 9.0~12.6% lignin and 9.1~17.9% hemicellulose remained in the residual biomass. However, the pretreated biomass did not lose much cellulose content, from 66.4 g to 72.9 g per 100 g of dry soybean straw, with an increase in the sodium hydroxide concentration. The delignification yield with the alkali-thermal pretreatment was 34.1~53.0%, in proportion to the amount of sodium hydroxide, from 0.5 to 3.0 M. However, >47% of the lignin could not be removed from the biomass under the alkali-thermal pretreatment conditions, even with a high concentration of sodium hydroxide. Nevertheless, the alkali-thermal treatment of the biomass showed that sodium hydroxide reduces hemicellulose and lignin effectively and increases the cellulose content per gram biomass.

3.2. Enzymatic Hydrolysis of Soybean Straw. To assess the enzymatic hydrolysis of alkali-thermal-pretreated soybean
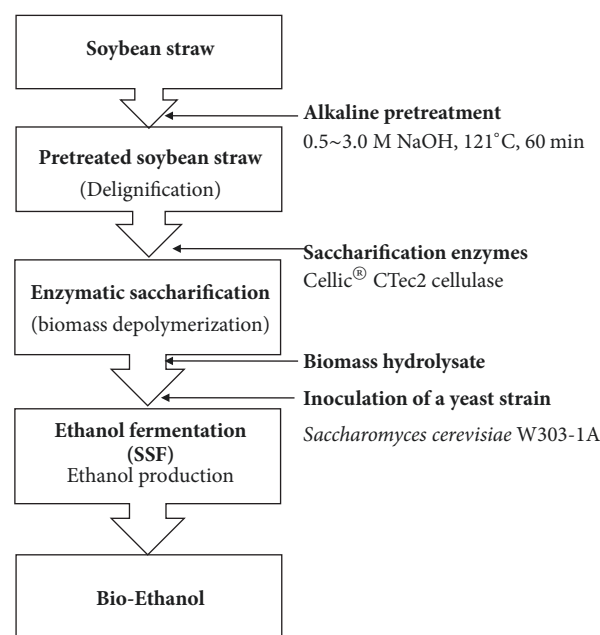

FIGURE 1: Overall procedure for the simultaneous saccharification and fermentation of the alkali-treated soybean straw for ethanol production.

straw with 2 M sodium hydroxide, the pretreated biomass (10% (w/v)) was digested with different enzyme dose of Cellic CTec2 cellulase, from 10 to 50 FPU per gram of dry biomass in phosphate buffer (pH 6.0) for 72 h. The pH in the reaction media for the enzymatic digestion could affect the growth, metabolism, and transport in an ethanologenic strain for further ethanol fermentation [15]. Thus pH was fixed at 6.0 for the enzymatic biomass saccharification. The amount of glucose produced by enzymatic hydrolysis increased with increasing amounts of cellulase per unit the alkali-pretreated soybean straw (Figure 2(a)). At 72 h, the cellulose generated 40.1~67.3 g/L glucose and 5.6~9.4 g/L xylose. The enzymatic digestibility at the enzyme loading ratio of 50 FPU cellulase per gram of dry biomass reached 54.2 ± 2.8%~90.9 ± 2.8% (Figure 2(b)). On the other hand, the nontreated biomass was hydrolyzed with less than 33% (data now shown). Compared with untreated soybean straw, the alkali-thermal-pretreated biomass contained higher amounts of glucose and lower amounts of xylose. Alkali-pretreatment with approximate

Table 2: Effect of the concentration of sodium hydroxide for the alkali-pretreatment of the soybean straw and the efficiency of delignification.

| NaOH | Soluble[2] | Insoluble[2] | Compositions/100 g soybean straw[1] | | | | | Delignification[4] |
| | | | Cellulose | | Hemicellulose | | Lignin[3] | |
[M]	[g]	[g]	[g]	[%][5]	[g]	[%][5]	[g]	[%]
Untreated soybean straw	-	100.0 ± 0.8	44.2 ± 0.6	44.2	5.9 ± 0.5	5.9	19.2 ± 0.7	-
0.0 M	29.1 ± 0.5	70.9 ± 0.7	36.8 ± 0.8	51.9	5.7 ± 0.6	8.1	17.9 ± 0.5	6.9
0.5 M	52.0 ± 0.4	48.0 ± 0.5	31.9 ± 0.5	66.4	8.6 ± 0.7	17.9	12.6 ± 0.6	34.1
1.0 M	55.0 ± 0.3	45.0 ± 0.6	30.8 ± 0.7	68.6	6.9 ± 0.4	15.4	12.2 ± 0.5	36.5
1.5 M	57.5 ± 0.3	42.5 ± 0.5	30.0 ± 0.6	70.7	5.4 ± 0.2	12.7	11.9 ± 0.4	38.1
2.0 M	60.3 ± 0.6	39.7 ± 0.7	29.4 ± 0.6	74.0	4.1 ± 0.3	10.3	10.1 ± 0.6	47.4
2.5 M	62.8 ± 0.7	37.2 ± 1.2	27.4 ± 0.4	73.7	3.6 ± 0.2	9.7	10.1 ± 0.4	47.3
3.0 M	64.5 ± 1.2	35.5 ± 0.7	25.8 ± 0.5	72.9	3.2 ± 0.2	9.1	9.0 ± 0.4	50.0

[1]100 g soybean straw is the weight of the total solid without the moisture content. [2]Soluble and insoluble indicate soluble and insoluble solids in the total solids in soybean straw biomass. [3]The lignin content is the sum of acid-soluble and acid-insoluble lignin. [4]The percent of the relative delignification was calculated by the following formula: delignification (%) = $100 \times (1 - ([lignin]_{treated\ soybean\ straw}/[lignin]_{untreated\ soybean\ straw}))$. [5]The percent of each component means the relative amount in insoluble fraction.

(a)

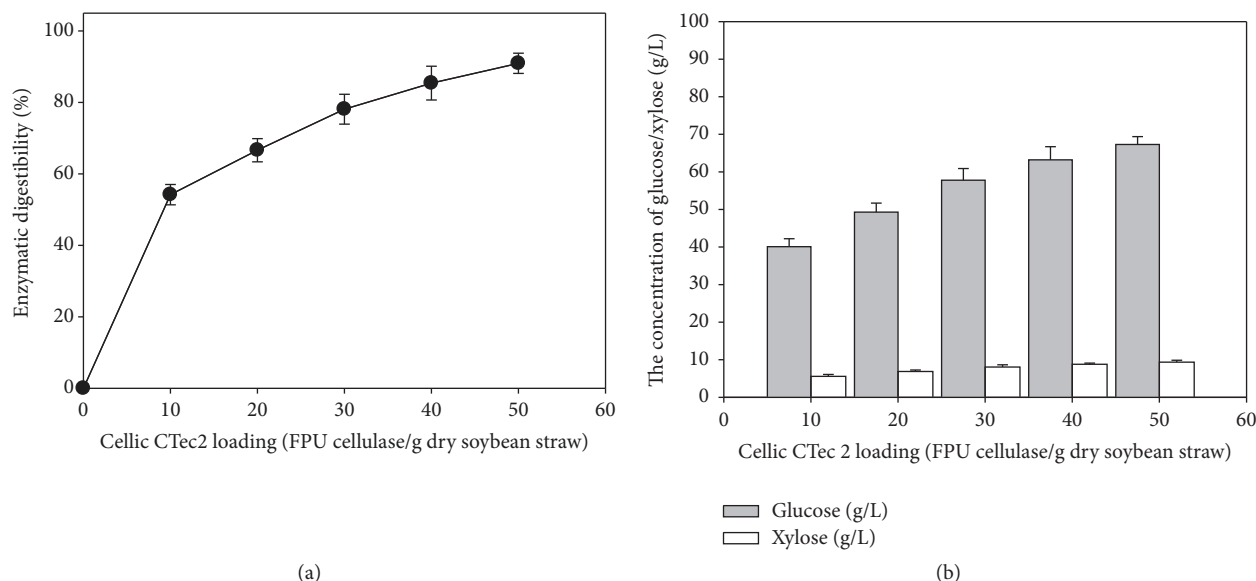Glucose (g/L)
Xylose (g/L)

(b)

FIGURE 2: Enzymatic saccharification of the alkali-treated soybean straw with 50 FPU of Cellic CTec2 cellulase loading per gram of the dry biomass. The enzymatic digestibility was calculated by the relative amount of glucose released from the content of glucan in the pretreated biomasses after enzyme reaction.

47.4% delignification efficiency would affect the cellulase digestibility, resulting in a hydrolysis efficiency up to 90%. The chemical pretreatment also reduced the strong interactions in cellulose/hemicellulose/lignin complexes in the biomass structure, enhancing the enzyme reaction efficiency. An increase in the volume of enzymes used significantly affects the amount of fermentable sugar generation. At the final reaction time point, the amount of glucose increased up to 1.7 times depending on 5 enzyme loading ratios of Cellic CTec2 cellulase per biomass. Additionally, sugar production increased time-dependently, whereas enzymatic hydrolysis rates decreased exponentially due to product inhibition (data not shown). The generated glucose, xylose, and incompletely digested cellobiose may inhibit the hydrolysis reactions of the cellulase. These enzymatic saccharifications showed that alkali-pretreated soybean straw could be powerful to prepare a fermentable sugar glucose for bioethanol production.

3.3. Separate Hydrolysis and Fermentation of Alkali-Pretreated Soybean Straw.

To evaluate the fermentability of the sugars generated from the alkali-pretreated soybean straw, ethanol production was performed using *Saccharomyces cerevisiae* W303-1A [12]. Batch cultivation of the yeast strain was performed in a 50-mL culture volume in a 250-mL Erlenmeyer flask with 5 g of alkali-pretreated soybean straw, supplemented with a 50 FPU Cellic CTec2 cellulase. Before cell inoculation, prehydrolysis was performed at 42°C for 12 h. Then, 5% (v/v) yeast inoculum was added and cultured further (30°C, 180 rpm, 36 h) for ethanol fermentation. The prehydrolysis step generated ~12.1 ± 0.7 g/L glucose in the flask for yeast cell growth without the lag phase. At 36 h, the maximum ethanol concentration of 13.1 ± 0.7 g/L was
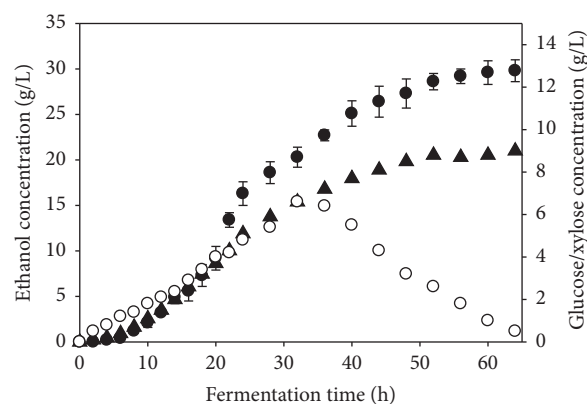

FIGURE 3: Batch simultaneous saccharification and fermentation of the alkali-pretreated soybean straw by *Saccharomyces cerevisiae* W303-1A in a 1-L jar fermenter. Profiles of ethanol (filled circle), glucose (open circle), and xylose (filled triangle) in the fermenter were analyzed by HPLC.

observed in the culture broth. However, low ethanol production yield was observed in the flask culture. The enzymatic saccharification was not efficient in the flask culture.

3.4. Batch Simultaneous Saccharification and Fermentation (SSF) for Ethanol Production.

To increase ethanol production, ethanol fermentation was performed in a 1-L jar fermentor. For batch SSF, 10% (w/v) pretreated biomass was enzymatically saccharified with 50 FPU of Cellic CTec2 cellulase per gram of dry biomass. The ethanol production proceeded gradually for 72 h (Figure 3). The amount of glucose was gradually increased by enzymatic hydrolysis. Although the ethanologenic yeast consumed glucose for cell growth, the

concentration of glucose generated exceeded the amount consumed. The yeast completely consumed all glucose enzymatically hydrolyzed from the biomass in the medium over 60 h. Nevertheless, the amount of xylose increased gradually to 9.1 ± 0.5 g/L, because the wild type *S. cerevisiae* W303-1A can not utilize the sugar. In a batch SSF, the maximum ethanol concentration was 30.5 ± 1.2 g/L at 72 h, giving an ethanol production yield of 0.305 g ethanol/g dry soybean straw and 0.45 g ethanol/g glucose at 72 h. Finally, 82% of the fermentable sugar hydrolyzed from the pretreated biomass was used by the yeast for ethanol production. In addition, the decrease of ethanol concentration was not observed until glucose was completely depleted in the fermentation (72 h).

In ethanol production of the alkali-pretreated soybean straw, the baker's *S. cerevisiae* W303-1A generated minor metabolites, including glycerol and organic acids such as acetic acid, lactic acid, and succinic acid (data not shown). These metabolites are less than 1.5 ± 0.4 g/L. These minor byproducts generated by the yeast were at lower concentrations than ethanol. Although these metabolites remained and were accumulated during the ethanol production, it did not affect the fermentation efficiency or the yeast cell growth.

In this study, an alkali-pretreatment was applied to an agricultural residue soybean straw as a potential bioresource to produce fermentable sugars by enzymatic saccharification. The soybean straw used in this pretreatment contained relatively high cellulose and lignin contents rather than hemicellulose. To reduce lignin and to enhance cellulose content, sodium hydroxide among chemical pretreatments was chosen for delignification of soybean straw. The maximum cellulose content of 74% was achieved with 2 M NaOH at 121°C after 60 min and, at this condition, delignification of 47.4% and hemicellulose content of 10.3% (Table 2).

In fact, the advantage of sodium hydroxide pretreatment can break down effectively the internal structure of lignocellulose component through degrading the ester and glycosidic chains. In addition, the alkali can alter the structure of lignin, causing cellulose swelling and partial decrystallization of cellulose [16]. Moreover the safer handling and the lower toxicity in the pretreatment, including lower operating temperatures and faster process times, make it an attractive alternative to ammonia pretreatment [17]. Relatively lower thermal-pretreatment condition also reduce sugar degradation products such as furfural, hydroxymethylfurfural, and organic acids, considering potential inhibitors for enzymatic saccharification as well as ethanol fermentation [18]. In enzymatic digestion and the fermentation process, these inhibitors were not detected in the biomass lysate. Moreover, the soybean straw pretreated with 2 M NaOH was effectively hydrolyzed with a hydrolysis efficiency up to 90% for production of the fermentable sugars (Figure 2(a)). Additionally, the simultaneous saccharification and fermentation converted the sugar of the biomass hydrolysate to 30.5 g/L of ethanol with ethanol conversion of more than 82% (Figure 3).

Although the concentration of sodium hydroxide for pretreatment of soybean straw was relatively higher than other alkali chemical compounds, sodium hydroxide solution could be recovered and reused several times for biomass pretreatment [19]. In addition, compared with other chemical pretreatments using aqueous ammonia, organosolv, and ionic liquids, the sodium hydroxide-treatment could be a simple and convenient process, because it does not require any special equipment or control systems for the pretreatment [19].

The utilization of soybean straw could offer several advantages for the sustainable development based on the biomass utilization. Sugar crops or alternative lignocellulosic biomass plants consumed nutrients in the soil leading to decreased nutrients levels. On the other hand, a legume plant soybean with symbiotic bacteria Rhizobia in the nodules of its root systems can fix nitrogen into ammonia and ammonium leading to nitrogen (N) enrichment in the soil, including CO_2 fixation by photosynthesis [20]. Considering the sustainability, the residual biomass from the soybean is a potential resource for production of fermentable sugar.

4. Conclusions

The alkali-pretreatment of soybean straw with sodium hydroxide effectively removed lignin and hemicellulose and enhanced enzyme digestibility. The alkali-pretreated biomass showed ~53% delignification efficiency. In the pretreated biomass, over 90% of the cellulose was hydrolyzed by Cellic CTec2 cellulase and was converted to fermentable sugars during enzymatic saccharification. In a flask-scale culture of separate hydrolysis and fermentation supplemented with the pretreated biomass, *S. cerevisiae* produced 13.1 g/L ethanol with 0.13 g ethanol/g biomass. In a batch of simultaneous hydrolysis and fermentation, the pretreated soybean straw was converted to 30.5 g/L of ethanol with the product yield of 0.305 g ethanol/g dry soybean straw and 0.45 g ethanol/g glucose. In the fermentation, 82% fermentable sugar was converted to ethanol within a low concentration of organic acid byproducts. These results clearly show that alkali-pretreatment for an agricultural byproduct soybean straw efficiently reduces lignin and hemicellulose components and increases enzymatic digestibility and ethanol productivity.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this article.

Acknowledgments

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