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Chapter 4

Effectiveness of Lignin-Removal in Simultaneous Saccharification and Fermentation for Ethanol Production from Napiergrass, Rice Straw, Silvergrass, and Bamboo with Different Lignin-Contents

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1. Introduction

Second-generation biofuels from lignocellulosic materials have gained much attention since the lignocelluloses are not in competition with food sources and animal feed and will provide a new sustainable energy sources alternative to petroleum-based fuels (Galbe and Zacchi, 2007). Bioethanol production from herbaceous lignocellulose such as corn stover (Ryu and Karim, 2011), rice straw (Ko *at al*., 2009), sweet sorghum bagasse (Cardoba *et al*., 2010), switchgrass (Keshwani and Cheng, 2009), bamboo (Sathitsuksanoh *at al*., 2010), wheat straw (Talebnia *et al*., 2010), alfalfa stems (González-García *at al*., 2010), and silvergrass (Guo *et al*., 2008) has been extensively developed through a variety of processes combining the biological saccharification and fermentation steps with the pre-treatment methods. In almost all processes, the pretreatments to remove the lignin components and to promote an enzymatic digestibility of cellulosic components are carried out by the use of energy and cost which are frequently higher than those of bio-fuels gained (Alvira *et al*., 2010). If lignocelluloses with low lignin-content are selected, the operation to remove the lignin might be excluded from the bio-ethanol process.

Among the many kinds of lignocelluloses, therefore, we (Yasuda *et al*., 2011; Yasuda *et al*., 2012) and other groups (Li *et a*l., 2011; Brandon *et al*., 2011; Zhang *et a*l., 2011; Huang *et al*.,

2011; Lin *et al*., 2011a; Lin *et al*., 2010b; Kai *et al*., 2010; Anderson *et al*., 2008) have been interested in napiergrass (*Pennisetum purpureum* Schumach) which is herbaceous lignocellulose with its low lignin- content. During our investigations on bioethanol production, it was found that the alkali-pretreatment of napiergrass enhances scarcely the ethanol yield whereas the alkali-pretreatment of silvergrass (*Miscanthus sinensis* Anderss) remarkably enhances the ethanol yield (Yasuda *et al*., 2011). Here, we compared the effectiveness of lignin-removal between napiergrass and other lingocelluloses with different lignin-contents (rice straw, silvergrass, and bamboo) in order to evaluate the availability of non-pretreated napiergrass as the raw materials of bio-ethanol.

2. Materials and methods

2.1. Chemical components of herbaceous lignocellulose

The lignocellulosic materials were cut, dried, and powdered until the 70 % of the particles became in a range of 32-150 μm in length to promote the cellulase- saccharification and to reduce varying in components in each experiment. The lignin-contents in lignocelluloses were determined as follows. The powdered lignocelluloses (30.0 g) was washed with MeOH and treated with a 1% aqueous solution of NaOH (400 mL) at 95 ºC for 1 h (Silverstein, *et al.*, 2007; Yasuda *et al.*, 2011; Yasuda *et al.*, 2012). After centrifugation at 10,000 rpm for 10 min to separate the precipitates, the supernatant solution was neutralized to pH 5.0 by a dilute HCl solution to give the lignin as a dark brown precipitate. The lignin-contents of napiergrass, rice straw, silvergrass, and bamboo were determined to be 14.9, 18.2, 21.7, and 26.2 wt%, respectively.

The holocellulose (cellulose and hemicellulose) was isolated as a pale yellow precipitate by the above centrifugation. The saccharide components of holocellulose were determined according to the methods published by the National Renewable Energy Laboratory (NREL) as follows (Sluiter *et al.*, 2010). Sulfuric acid (72%) was added to holocellulose and then diluted with water until the concentration of sulfuric acid became 4%. This was heated at 121 ºC for 1 h in a grass autoclave (miniclave, Büchi AG, Switerland). HPLC analysis of the hydrolyzate showed that holocellulose mainly composed of glucose and xylose along with the small amounts of arabinose and galactose. The ash component in lignocelluloses was obtained by the burning of the lignocelluloses (2.0 g) in an electric furnace (KBF784N1, Koyo, Nara, Japan) for 2 h at 850 ºC. Chemical components of lignocelluloses are shown in Table 1.

2.2. Saccarification

As has been previously reported (Yasuda *et al*., 2011; Yasuda *et al*., 2012), a cellulase from *Acremonium cellulolyticum* (Acremozyme, Kyowa Kasei, Osaka, Japan) was selected by the comparison in activity with other cellulase such as Meycellase (Kyowa Kasei), a cellulase from *Trichoderma viride* (Wako Chemicals, Osaka, Japan) and a cellulase from *Aspergillus niger* (Fluka Japan, Tokyo). The cellulase activity of Acremozyme was determined by the method of the breakdown of filter paper (Yasuda *et al*., 2012). At first, cellulase activity was

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defined as 10,000 units when two sheets of filter papers (1 cm×1 cm) degraded at pH 5.0 and 45 °C by the cellulase for 150 min. The filter papers were entirely degraded in 114 min by 10 mg of Acremozyme. Thus, cellulase activity of Acremozyme was determined to be 1320 units mg^{-1} according to the following equation: cellulase activity (units mg^{-1}) = 150×10,000/(a×b) where a and b denoted weight of cellulase in mg and period in min required for the degradation, respectively.

Lignocelloloses	Components/g [a)]			
	Holocellulose (hexose : pentose) [b)]	Lignin	Ash	Others
Napiergrass [c)]	57.3 (37.5 : 26.5)	14.9	12.7	15.1
Rice straw	61.3 (39.7 : 28.4)	18.2	17.7	2.8
Silvergrass	41.0 (34.2 : 11.4)	21.7	4.0	33.3
Bamboo	66.5 (43.9 : 30.0)	26.2	1.4	5.9

a) The amounts of components derived from 100 g of lignocellulose.

b) The values in the parenthesis are the amounts (g) of hexose and pentose derived from 100 g of lignocelluloses.

c) Referred from Yasuda *et al*., 2012.

Table 1. Components of herbaceous lignocellolosic materials

The saccharification of the powdered cellulosic materials (10.0 g) was performed with Acremozyme (1.0 g) in an acetate buffer (60 mL, pH 5.0) under vigorous shaking at 45 °C. At the given saccharification time, the portion was taken from the reaction mixture and centrifuged at 12,000 rpm. The supernatant solutions were subjected to analysis for saccharides. The amounts of the reducing saccharides obtained from the saccharification reactions at 30, 40, and 45 °C were almost the same.

2.3. Simultaneous Saccharification and Fermentation (SSF)

Saccharomyces cerevisiae NBRC 2044 was grown at 30 ºC for 24 h in a basal medium (initial pH 5.5) consisting of glucose (20.0 g L^{-1}), peptone (1.0 g L^{-1}, Difco), yeast extract (1.0 g L^{-1}), $NaHPO_4$ (1.0 g L^{-1}), and $MgSO_4$ (3.0 g L^{-1}). After incubation for 24 h, the cell suspension of *S. cerevisiae* was obtained. The grown culture of *S. cerevisiae* showed a cell density of 7.7×10^7 cells mL^{-1}.

The suspension of cellulosic materials (1.33 g) in an acetate buffer solution (5 mL, pH 5.0) was introduced into the test tube (100 mL) and was autoclaved at 121 ºC for 20 min. After cooling the autoclaved suspension of cellulosic materials, the cell suspension (0.16 mL) of *S. cerevisiae* and the Acremozyme cellulase (133 mg) in an acetate buffer solution (3 mL, pH 5.0) were added (Yasuda *et al*., 2012). The glucan contents were determined to be 436, 475, 410, and 525 mg in non-treated cellulosic materials (1.33 g) of napiergrass, rice straw, silvergrass, and bamboo, respectively. In the case of alkali-treated cellulosic materials (1.33 g), 761 (na-

piergrass), 774 (rice straw), 999 (silvergrass), and 790 mg (bamboo) of the glucan contents were included. The reaction vessel was connected by tube to messcylinder set in a water-bath to collect the evolved CO_2 gas. The reaction progress was monitored by the volume of CO_2. Thus, the simultaneous saccharification and fermentation (SSF) process was performed by stirring vigorously the reaction mixture with a magnetic stirrer at 34 °C, which is the optimal temperature.

2.4. Analysis

Saccharides were analyzed on a high-performance liquid chromatography system (LC-20AD, Shimadzu, Kyoto, Japan) equipped with RI detector (RID-10A) using anion exchange column (NH2P-50 4E; Shodex Asahipak, 250 mm in length and 4.6 mm in ID, Yokohama, Japan). Acetonitrile-water (8:2 v/v) was flowed at 1.0 mL min^{-1} as mobile phase. As a method to supplement LC analysis of saccarides, the amount of the reducing sugars released by the saccharification process was analyzed by a modified Somogyi–Nelson method (Kim and Sakano, 1996) assuming the composition of sugars to be $C_6H_{12}O_6$. The amounts of pentose were analyzed by a modified orcinol method using 5-methylresorcinol (orcinol), $FeCl_3$ $5H_2O$, and conc HCl (Fernell and King, 1953). Ethanol was analyzed by gas-liquid chromatography using a Shimadzu gas chromatograph (model GC–2014) and a glass column of 5% Thermon 1000 on Sunpak-A (Shimadzu) with 2-propanol as an internal standard. Scanning electron microscope (SEM) images were taken on a Hitachi S–4100 (Tokyo, Japan).

3. Results and discussion

3.1. Napiergrass (*Pennisetum purpureum* Schumach)

Napiergrass is a herbaceous tropical species, native to the east Africa. There are wide variation of phenotypes in napiergrass, reflected by plant breeding due to the crossing of dwarf genotype and relative species such as pearl millet (*Pennisetum americanum*) (Ishii *et al*., 2005a, Hanna and Sollenburger, 2007). Dwarf variety of late-heading type originated from Florida, USA, via Thailand (Mukhtar *et al*., 2003) was assessed to be suitable for both grazing (Ishii *et al*., 2005b) and cut-and-carry systems among several sites of southern Kyushu, Japan (Utamy *et al*., 2011). Dwarf variety of napiergrass meets the requirement of lignocellulose for the biofuel production, because it has low lignin-content and a high herbage mass per year and per area (Rengsirikul *et al*., 2011). Therefore, we have continued to use this dwarf type of napiergrass for the bio-ethanol (Yasuda *et al*., 2011) and bio-hydrogen production (Shiragami *et al.*, 2012) in University of Miyazaki.

3.2. Alkali-pretreatment

The powdered lignocelluloses (30.0 g) were washed with MeOH to remove lipids and treated with a 1% aqueous solution of NaOH (400 mL) at 95 ºC for 1 h (Silverstein, *et al.*, 2007).

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The resulting lignin-removed holocellulose was isolated by centrifugation of the solution at 10,000 rpm for 10 min. Lignin remained in the alkali solution. The precipitate was washed by dispersion in water to remove the contaminated lignin. After the pH-adjustment to 7.0, the washed holocellulose was collected by centrifugation and dried.

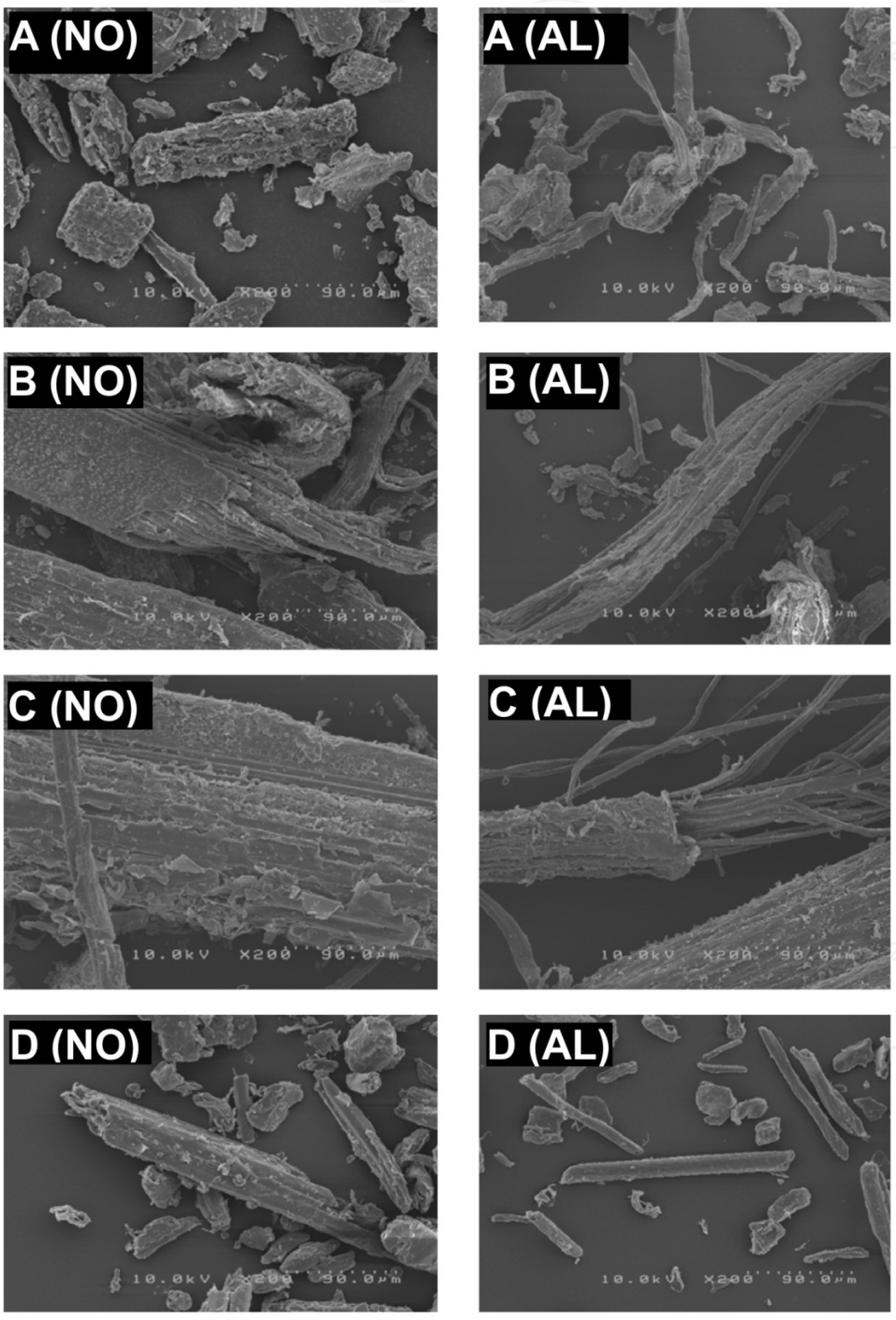

Figure 1. SEM images of non-treated (NO) and alkali-pretreated (AL) napiergrass (A), rice straw (B), silvergrass (C), and bamboo (D). The SEM images were taken under the magnification of 200.

Physical changes from non-pretreated lignocelluloses to alkali-pretreated lignocelluloses were studied using SEM images, as shown in Fig. 1. The fiber bundles observed in lignocelluloses were unloosened by the removal of lignin to change into the thin fibers in the alkali-pretreated lignocelluloses. It was expected that the accessibility of enzyme to the cellulose was increased by the alkali- pretreatment.

3.3. Lignin-removal effect on saccharification

The saccharification of alkali-pretreated lignocelluloses (holocellulose, 10.0 g) was performed with Acremozyme (1.0 g) in an acetate buffer (60 mL, pH 5.0) under vigorous shaking at 45 °C. The amounts of saccharides obtained from 1 g of alkali-pretreated napiergrass, rice straw, silvergrass, and bamboo were transformed to the amounts per 1.0 g of the alkali-untreated samples by multiplication with 0.573, 0.613, 0.410, and 0.665 g g^{-1} which were the contents of holocellulose. Table 2 summarizes the amounts of hexose and pentose after the saccharification reaction for the time (T_{SA}) to reach the maximum yields. In the cases of napiergrass and rice straw, the hexose yields (87.5 and 81.9 %) reached almost maximum yields whereas the pentose yields were still low. The largest amount of reducing saccharide was 451 mg obtained from 1.0 g of rice straw.

In order to examine the effectiveness of alkali-pretreatment, the saccharification of the non-pretreated lignocelluloses (10.0 g) was performed under conditions similar to the case of alkali-pretreated lignocelluloses. The largest amount of reducing saccharide was 307 mg g^{-1} obtained from non-pretreated napiergrass. Figure 2 shows the time-conversions of the saccharification reactions of non-pretreated and alkali-pretreated lignocelluloses. In all cases, the yields of saccharides from the alkali-pretreated lignocelluloses were higher than those from the non-pretreated lignocelluloses. The ratios (E_{SA}) of saccharide yields from the alkali-pretreated lignocelluloses to those from the non-pretreated lignocelluloses were used as a measure of the effectiveness of the lignin-removal on the saccharification process. The E_{SA} values are listed in Table 2.

3.4. Effectiveness of lignin-removal on Simultaneous Saccharification and Fermentation (SSF)

Ethanol was produced through a simultaneous saccharification and fermentation process (SSF) under optimal conditions as follows (Yasuda, *et al*., 2012). Acremozyme (133 mg) in an acetate buffer solution (3.0 mL, pH 5.0) and the cell suspension (0.16 mL) of *S. cerevisiae* were added to the suspension of alkali-pretreated lignocelluloses (1.33 g) in an acetate buffer solution (5.0 mL, pH 5.0). The mixture was reacted at 35 °C under vigorous stirring until the CO_2 evolution ceased. The amounts of the products were transformed to the amounts per 1.0 g of the alkali-unpretreated lignocelluloses by the dividing by 1.33 and multiplication with 0.573 (napiergrass), 0.613 (rice straw), 0.410 (silvergrass), and 0.665 g g^{-1} (bamboo). Table 3 lists the amounts of ethanol and the recovered hexose and pentose which were determined by averaging the data of seven experiments. The maximum ethanol yield in SSF of alkali-pretreated lignocelluloses was 139 mg g^{-1} from rice straw.

Lignocelluloses	PT[a)]	T_{SA}/h [b)]	Product [c)]/mg g^{-1} (Yield/%) [d)]			E_{SA}
			Hexose	Pentose	Total	
Napiergrass	NO	120	215 (57.3)	91 (34.3)	307 (48.1)	1.36
	AL	120	328 (87.5)	90 (34.0)	419 (65.7)	
Rice straw	NO	120	192 (48.4)	51 (18.0)	244 (35.8)	1.85
	AL	120	325 (81.9)	125 (44.0)	451 (66.2)	
Silvergrass	NO	120	122 (35.7)	39 (34.2)	161 (35.3)	1.57
	AL	120	178 (52.0)	75 (65.8)	253 (55.5)	
Bamboo	NO	120	69 (15.7)	19 (6.3)	88 (11.9)	3.39
	AL	120	180 (41.0)	118 (39.3)	297 (40.2)	

a) Pretreatment (PT). NO: non-treatment, AL: lignin removal by alkali-pretreatment.

b) Saccharification time when the total yield of saccharides reached the maximum.

c) The amounts of products per 1 g of lignocellulosewhen the total yield of saccharides reached the maximum.

d) Yields were based on the amounts of hexose and pentose occurring in lignocelluloses.

Table 2. The lignin removal effects on saccharification processe

Lignocelluloses (EtOH/mg g^{-1}) [a)]	PT [b)]	T_{SSF}/h [c)]	Product [d)]/mg g^{-1}			E_{SSF}
			Hexose	Pentose	EtOH (Yield/%) [e)]	
Napiergrass (192)	NO	24	18±5.2	99±1.6	102±3.5 (53.2)	1.18
	AL	96	38±5.3	125±5.0	121±4.6 (63.1)	
Rice straw (203)	NO	24	20±8.0	102±6.5	96±5.9 (47.3)	1.45
	AL	192	27±7.2	152±6.2	139±1.4 (68.5)	
Silvergrass (175)	NO	24	13±2.2	48±7.4	41±9.4 (23.5)	1.77
	AL	96	12±3.4	93±3.5	72±4.3 (41.2)	
Bamboo (224)	NO	24	6±5.1	18±5.8	34±1.7 (15.2)	2.28
	AL	96	22±4.3	111±1.5	78±5.6 (34.8)	

a) Theoretical amounts of ethanol obtained from glucan in lignocellulose (1 g).

b) Pretreatment (PT). NO: non-treatment, AL: lignin removal by alkali-pretreatment.

c) SSF time until the CO_2 evolution ceased.

d) The amounts of products per 1 g of lignocellulosewhen the SSF reaction reached the maximum. Data were determined by averaging the data of seven experiments.

e) Yield of ethanol based on the amounts of hexose occurring in lignocelluloses.

Table 3. The lignin removal effects on SSF processe

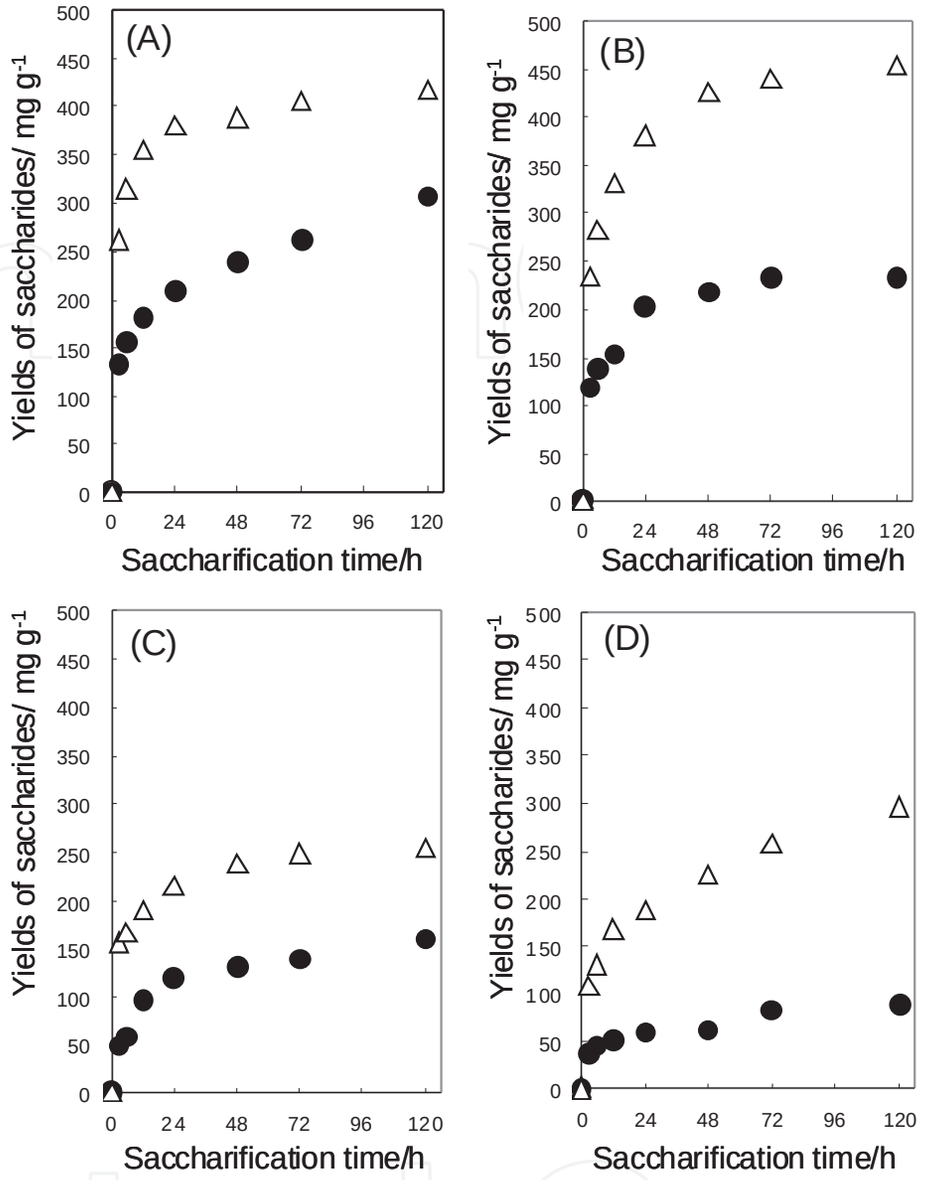

Figure 2. Time conversion of the saccharification of napiergrass (A), rice straw (B), silvergrass (C), and bamboo (D) for the non-pretreated lignocelluloses (●) and the alkali-pretreated lignocelluloses (△). The amounts of sugar from the alkali-pretreated lignocelluloses were transformed to the amounts per 1 g of the alkali-unpretreated samples by multiplication with 0.573 (napiergrass), 0.613 (rice straw), 0.410 (silvergrass), and 0.665 g g^{-1} (bamboo).

After the SSF, the pentose remained in the solution, although the hexose was consumed by the fermentation with *S. cerevisiae*. The amounts of pentose was compared between SSF and cellulase-saccharification processes under the optimized conditions. The amounts of pentose formed in SSF were larger than those in saccharification, except for the case of bamboo (Table 2 and 3). Therefore, the SSF process accelerated the hydrolysis of cellulosic components compared to the saccharification process. The consumption of saccharides by fermentation with *S. cerevisiae* might move the equilibrium to the product side in the hydrolysis of cellulosic components to saccharides with Acremozyme. In the case of bamboo, the ethanol yield

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was low, irrespective of higher content of hexose probably because of poor accessibility of the enzyme to holocellulosic components of bamboo (Yamashita *et al*., 2010).

Also, the SSF process was applied to the non-pretreated lignocelluloses. The time- conversions of CO_2-evolution were compared between non-pretreated and the alkali-pretreated lignocelluloses, as shown in Fig. 3. The yields of ethanol from non- pretreated lignocelluloses were lower compared with the cases from alkali-pretreated lignocelluloses. Among the non-pretreated lignocelluloses, the largest amount of ethanol was 102 mg g^{-1} obtained from napiergrass. The enhanced effect of SSF yields by alkali-pretreatment was evaluated by the ratio (E_{SSF}) of ethanol yields from the alkali-pretreated lignocelluloses to those from non-pretreated lignocelluloses. The E_{SSF} values are listed in Table 3.

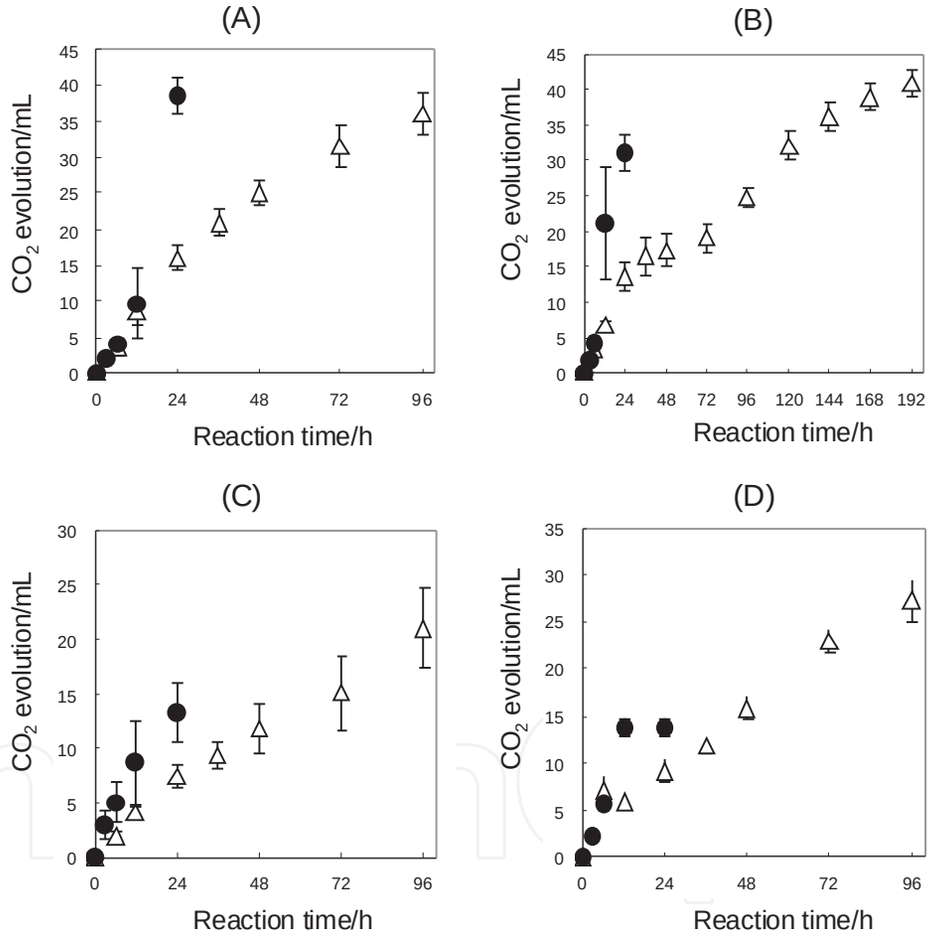

Figure 3. CO_2-evolution in the SSF of napiergrass (A), rice straw (B), silvergrass (C), and bamboo (D) for the non-treated lignocelluloses (●) and the alkali-pretreated lignocelluloses (△). The amounts of CO_2 from alkali-pretreated lignocelluloses was transformed to the amounts per 1 g of the alkali-unpretreated samples by multiplication with 0.573 (napiergrass), 0.613 (rice straw), 0.410 (silvergrass), and 0.665 g g^{-1} (bamboo).

It is noteworthy that the SSF of alkali-pretreated lignocelluloses was remarkably slowed down in all cases. In the fermentation by *S. cerevisiae* of the alkali-pretreated lignocelluloses, a nitrogen-source and a mineral were thought to be insufficient, since the aminoacids and

the mineral were removed from lignocelluloses by alkali-pretreatment and the additional nutrients were not added in the SSF process (Alfenore *et al*., 2003). Moreover, the fermentation process was affected by the inhibitory materials derived from the alkali-pretreatment since T_{SA} of both non-pretreated and the alkali-pretreated lignocelluloses were almost same (Alvira, 2010).

3.5. Availability of napiergrass as raw materials for ethanol production

In the cases of rice straw, silvergrass, and bamboo with relatively high lignin-contents (18.2–26.2 wt%), the lignin-removal was effective for both saccharification and SSF processes because of the larger E_{SA} (1.57–3.39) and E_{SSF} values (1.45–2.28). However, in the case of napiergrass with low lignin-content (14.9 wt%), the E_{SSF} value was small (1.18). Figure 4 shows the plots of the E_{SSF} values against the lignin-contents of lignocelluloses. As the lignin-contents increased, the E_{SSF} values gradually increased. From the extrapolation of a fitting line of the plots, it is assumed that the E_{SSF} values at 13.4 wt% of lignin-content will reach 1.0 which means no enhancement effect of lignin-removal. Thus, it was elucidated that the alkali-treatment was effective for lignocelluloses with higher lignin content than 13.4 wt%, but was not effective as the pretreatment of lignocelluloses with lower lignin content than 13.4 wt%.

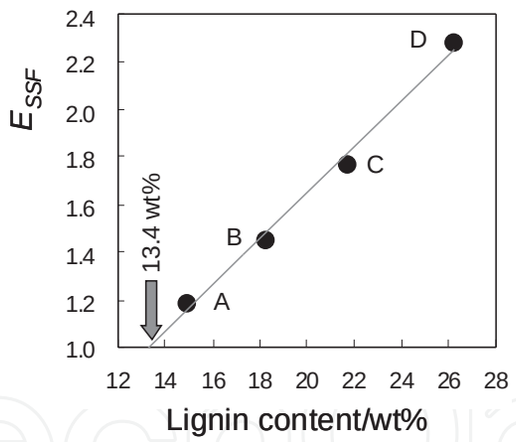

Figure 4. Dependence of E_{SSF} on the lignin contents in the SSF of napiergrass (A), rice straw (B), silvergrass (C), and bamboo (D). The plots showed that the E_{SSF} value became 1.0 at 13.4 wt% of lignin content.

4. Conclusion

In general, the alkali-pretreatment increases the accessibility of enzymes to the cellulose by the lignin-removal. Therefore alkali-pretreatment is effective for saccarification of the lignocellulose with higher lignin contents. In the case of napiegrass with low lignin- content,

ethanol was produced in 102 mg g^{-1} and 121 mg g^{-1} from napiergrass through the SSF without and with alkali-pretreatment, respectively. Taking into consideration the low effectiveness of lignin-removal in ethanol yield, the retardation of fermentation rate, the loss of nutrients for the fermentation by *S. cerevisiae*, and the cost of lignin-removal, we concluded that ethanol production from napiergrass should be performed through the SSF process without the alkali-pretreatment. For example, Inoue and his coworkers (Hideno *et al.*, 2009) have recently proposed the enzymatic saccharification of rice straw treated by a wet disk milling method without chemical pretreatment. Even so, the development of a pretreatment method with low energy and low cost to enhance saccharification yields by the structural change of cellulosic components rather than lignin-removal are desired for economically viable bio-ethanol production. In our group, the development of more efficient pretreatment method other than alkali-pretreatment to produce effectively bioethanol from napiergrass is now in progress.

Moreover, the fermentation of the pentose remaining in SSF is important subject. We (Yasuda *et al*., 2012) started the pentose fermentation using a recombinant *Escherichia coli* KO11. Pentose fermentation by *E. coli* KO11 produced additionally 31.4 mg g^{-1} of ethanol. Under the optimized conditions, the combination of the SSF and KO11 fermentation processes resulted in the production of 144 mg g^{-1} of ethanol from the non-pretreated napiergrass powder. The ethanol yield was 44.2% of the theoretical yield based on the hexose (375 mg) and pentose (265 mg) derived from 1 g of dry powdered napiergrass.

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