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Enhancement of Ethanol Production from Napiergrass (*Pennisetum purpureum* Schumach) by a Low-Moisture Anhydrous Ammonia Pretreatment

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ABSTRACT

Napiegrass (*Pennisetum purpureum* Schumach) was treated with a low-moisture anhydrous ammonia (LMAA) pretreatment by adding an equal weight of water and keeping it under atmospheric ammonia gas at room temperature for four weeks. After the removal of ammonia and washing with water, a simultaneous saccharification and fermentation (SSF) was conducted for the LMAA-pretreated napiergrass (1.33 g) in a buffer solution (8 mL) using a mixture of a cellulase (80 mg) and a xylanase (53 mg) as well as the cell suspension (0.16 mL) of *Saccharomyces cerevisiae*. Ethanol and xylose resulted in 91.2% and 62.9% yields, respectively. The SSF process was scaled up using LMAA-pretreated napiergrass (100.0 g) to give ethanol (77.2%) and xylose (52.8%). After the removal of ethanol, the pentose fermentation of the SSF solution (40 mL), which contained 1.00 g of xylose, using cell suspension of *Escherichia coli* KO11 (70 mL) gave 86.3% yield of ethanol. Total ethanol yield reached 68.9% based on xylan (21.4 wt%) and glucan (39.7 wt%) of the LMAA-pretreated napiergrass.

Keywords: LMAA; SSF; Cellulase; Xylanase; *Saccharomyces cerevisiae*; *Escherichia coli* KO11

1. Introduction

Biomass has gained much attention as a new sustainable energy source alternative to petroleum-based fuels [1]. The second-generation bioethanol from lignocellulosic materials became a promising approach since the lingocelluloses are not directly in competition with food sources. However, a variety of pretreatment methods to remove the lignin components and/or to promote an enzymatic digestibility of the cellulosic components have been required for efficient bio-ethanol production from lignocellulose [2].

It has been known that cellulose chains are incorporated into a number of distinct crystal phases each differing in chemical reactivity and material characteristics. The naturally occurring crystal phases, cellulose I [3] can be transformed into cellulose IIII by treating them with liquid ammonia [4] or various amines [5-9]. Therefore, the ammonia fiber explosion (AFEX) pretreatment was performed by heating the biomass with ammonia gas at 90˚C under 21 atm [10,11]. This was interpreted as an increase in the cellulose IIII phase which has a high reactivity toward enzymatic degradation [12]. Also, soaking in aqueous ammonia (SAA) at 40˚C - 80˚C in a room atmosphere was used as the pretreatment of lignocellulose [13,14]. Recently, Kim *et al.* [15] developed a pretreatment using gaseous ammonia, low-moisture anhydrous ammonia (LMAA) pretreatment, where the lingocellulose was kept in a flask filled with ammonia gas at 80˚C for 84 h.

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We were interested in ethanol production from herbaceous lignocellulosic napiergrass (*Pennisetum purpureum* Schumach) because of its low lignin content and high harvest amount per year and per area [16,17]. Ethanol was produced from non-pretreated napiergrass through simultaneous saccharification and fermentation (SSF) as well as pentose fermentation (PF) with recombinant *Escherichia coli* KO11 [18]. However, the total ethanol yield was still low (44.2%). Moreover, usual alkali-pretreatment did not operate effectively to enhance the ethanol yield [19]. Therefore, we applied a LMAA pretreatment to enhance the ethanol yield from napiergrass.

2. Materials and Methods

2.1. LMAA Pretreatment

The LMAA pretreatment was performed by modifying the Kim method [15]. Water (100 g) was added dropwise to the dry powdered napiergrass (100 g, volume 320 mL) in the flask (1 L). The resulting moist powdered napiergrass in the flask was evacuated with a pump under 20 mm Hg and then gaseous ammonia was introduced into the flask. This operation was performed three times until the atmosphere inside the flask was entirely replaced with ammonia. The amount of ammonia presented in the flask was 1.1 g. The moist powdered napiergrass was kept under ammonia gas atmosphere at room temperature for four weeks. After the treatment, the ammonia was removed with an evaporator. The treated napiergrass was washed with water (2 L) three times to separate the brownish aqueous solution of the lignin. The treated napiergrass was dried at 60˚C to weigh out the precise amount of napiergrass in the following biological treatment.

2.2. Chemical Components of Napiergrass

The powdered napiergrass (30 g) was treated with a 1% aqueous solution of NaOH (400 mL) at 95˚C for 1 h. The holocellulose was isolated as a pale yellow precipitate from the treated mixture by centrifugation and filtration. The supernatant solution was neutralized to pH 5.0 by a dilute HCl solution. The resulting dark brown precipitate identified as lignin was collected by centrifugation at 10,000 rpm for 10 min. Sugars in holocellolose were determined according to the methods published by the National Renewable Energy Laboratory (NREL) [20] as follows. Sulfuric acid (72 wt%, 3.0 mL) was added slowly to holocellulose (300 mg) and kept at 30˚C for 1 h. The resulting solution was diluted by water (84 mL) until the concentration of sulfuric acid was 4 wt%. Acid hydrolysis was performed by autoclaving at 121˚C for 1 h in an autoclave. After the neutralization by $CaCO_3$, the solution was subjected to the centrifugation to give the supernatant solution (ca. 87 mL), which was concentrated to 30 mL by evaporation. The solution was analyzed by HPLC. The peaks of glucose and xylose appeared whereas the peaks of galactose and arabinose were very weak because of their low contents. The amounts of glucan and xylan were determined from the amounts of glucose and xylose determined by HPLC. It was confirmed that the sum amounts of glucan and xylan were equaled to the amounts of hollocellolose. The ash component in lignocellulose was obtained by the burning of the lignocellulose (2.0 g) in an electric furnace (KBF784N1, Koyo, Nara, Japan) for 2 h at 850˚C. Thus, the chemical components of napiergrass were determined, as shown in **Table 1**.

2.3. Hydrolytic Enzyme

A cellulase from *Acremonium cellulolyticus* (Acremozyme KM, Kyowa Kasei, Osaka, Japan) was selected by comparing its activity with other cellulases such as Meycellase (Meiji Seika), a cellulase from *Trichoderma viride* (Wako Chemicals, Osaka, Japan) and a cellulase from *Aspergillus niger* (Fluka Japan, Tokyo) [18,21]. The cellulase activity of Acremozyme was determined to be 1320 units/mg by the method of breaking down filter paper [18]. A xylanases from *Trichoderma longibrachiatum* (*reesei*) (Sumizyme X, Shin Nihon Chemicals, Anjyo, Japan, 5000 u/g) was selected from commercially available hemicellulase.

2.4. Preparation of the Inoculum Culture of *Saccharomyces cerevisiae* and *Escherichia coli* KO11

Saccharomyces cerevisiae NBRC 2044 was grown at 30˚C for 24 h in a basal medium (initial pH 5.5) consisting of glucose (20.0 g/L), polypeptone (1.0 g/L), yeast extract (1.0 g/L), KH_2PO_4 (1.0 g/L), and $MgSO_4$ (3.0 g/L). After incubating for 24 h, the cell suspension of *S. cerevisiae* whose grown culture of *S. cerevisiae* showed a cell density of 7.7×10^7 cells/mL, was obtained [18]. *E. coli* KO11 was grown in the LB medium (200 mL) consisting of tryptone (2.0 g/L, Difco), yeast extract (1.0 g/L), and NaCl (2.0 g/L) under shaking at 150 rpm at 37˚C for 24 h. The KO11 cell suspension contained a dry weight of 0.52 mg/mL of *E. coli* KO11.

Table 1. Components of the NO- and LMAA-pretreated napiergrass.

PT[a)]	Components/wt%			
	Holocellulose (glucan : xylan)	Lignin	Ash	Others
NO[b)]	48.2 (31.3:16.9)	12.6	13.9	25.3
LMAA	61.1 (39.7:21.4)	7.1	7.1	24.7

[a)] Pretreatment (PT): NO: non-treatment. LMAA: low-moisture anhydrous ammonia pretreatment at room temperature for four weeks. [b)] The components of the LMAA-pretreatment napiergrass without washing with water was considered to be same as those of non-treated napiergrass.

2.5. Simultaneous Saccharification and Fermentation (SSF)

The LMAA-pretreated napiergrass (1.33 g) was suspended in the acetate buffer (5 mL, pH 5.0) and then autoclaved at 121˚C for 20 min. After cooling to room temperature under UV-irradiation, the cell suspension (0.16 mL) of *S. cerevisiae* and the hydrolytic enzyme (133 mg) in an acetate buffer solution (3.0 mL, pH 5.0) were added to the suspension of the LMAA-treated napiergrass [18]. After air was purged with N_2, the SSF was initiated by stirring the solution vigorously with a magnetic stirrer at an optimal temperature of 34˚C. The evolved CO_2 was collected over water by a messcylinder, and the reaction was monitored by the volume of CO_2. The SSF reaction was continued for 48 h until CO_2 evolution was ceased. **Table 2** lists the data expressed as averages of the experiments in three times.

2.6. Analysis

Saccharides were analyzed on a high-performance liquid chromatography system (LC-20AD, Shimadzu, Kyoto, Japan) equipped with RI detector (RID-10A) using an 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 as mobile phase. Ethanol was analyzed by gas-liquid chromatography using 2-propanol as an internal standard on a Shimadzu gas chromatograph (model GC-2014) equipped with a glass column of 5% Thermon 1000 on Sunpak-A (Shimadzu).

3. Results

3.1. Napiergrass as Lignocellulosic Materials

For the herbaceous lignocellulose, we selected a dwarf type of napiergrass (*Pennisetum purpureum* Schumach), which is a digestible tropical grass [16,17]. Napiergrass was cultivated in the Kibana Agricultural Science Station, at the University of Miyazaki. Leaf blades of the napiergrass were separated from the stem and then cut by a cutter and dried at 70˚C for 72 h. The dried matter was ground until the 70% of the particles were in a range of 32 - 150 μm in length.

3.2. Strategy for Ethanol Production from Napiergrass

In the case of lignocelluloses with high xylan contents, PF is an unavoidable process. In many cases, the PF using recombinant *E. coli* [22-24] or recombinant *S. cerevisiae* [25] has been incorporated with the process as cofermentation with hexose fermentation [15,26]. However, we performed the PF step separately, since HF using *S. cerevisiae* was well-established technique. Our process outline proceeded through SSF using a hydrolytic enzyme and *S. cerevisiae* followed by PF using *E. coli* KO11.

3.3. SSF of the LMAA-Pretreated Napiergrass

The results of the SSF process were summarized in **Table 2**. Without the pretreatment, the SSF using cellulase (Acremozyme KM) produced ethanol and xylose in

Table 2. The product yields in the SSF of napiergrass[a)].

Run	PT[c)]	F_X[d)]	Product/mg (Yield/%) [b)]			Ethanol/g L^{-1}[e)]
			Glucose	Xylose	Ethanol	
1[f)]	NO	0.0	13 ± 2 (2.8)	77 ± 29 (30.8)	122 ± 15 (51.6)	15.3
2	LMAA	0.0	32 ± 4 (5.5)	150 ± 14 (47.6)	179 ± 3 (59.7)	22.4
3	LMAA	0.1	25 ± 4 (4.2)	172 ± 5 (53.5)	182 ± 4 (60.5)	22.8
4	LMAA	0.2	20 ± 2 (3.5)	155 ± 14 (48.9)	197 ± 1 (65.7)	24.6
5	LMAA	0.3	22 ± 2 (3.7)	160 ± 3 (50.5)	219 ± 22 (72.9)	27.3
6	**LMAA**	**0.4**	**18 ± 10 (3.1)**	**199 ± 18 (62.9)**	**273 ± 7 (91.2)**	**34.2**
7	LMAA	0.5	14 ± 1 (2.3)	173 ± 2 (54.8)	260 ± 10 (86.5)	32.4
8	LMAA	0.6	23 ± 22 (4.0)	146 ± 5 (46.3)	197 ± 8 (65.5)	24.0
9	LMAA	0.7	26 ± 10 (4.4)	150 ± 9 (47.6)	198 ± 12 (66.0)	24.7
10[f)]	NO	0.4	142 ± 35 (30.7)	120 ± 23 (48.3)	98 ± 1 (41.3)	12.2
11[f)]	LMAA[g)]	0.4	34 ± 16 (7.4)	133 ± 6 (45.2)	178 ± 4 (75.1)	22.3

[a)] The SSF was performed for the pretreated napiergrass (1.33 g) in buffer solution (8 mL) containing hydrolytic enzyme (133 mg) and the cell suspension of *S. cerevisiae* (0.16 mL) at 34˚C for 48 h. The data were expressed as averages of three experiments. [b)] In the case of LMAA, the yields of glucose, xylose, and ethanol were calculated based on the theoretical amounts, 587 mg, 316 mg, and 300 mg, respectively; [c)] Pretreatment (PT). NO: non-treatment. LMAA: low-moisture anhydrous ammonia pretreatment at room temperature for four weeks. [d)] The F_X was the fraction of xylanase in the mixture (133 mg) of cellulase and xylanase. [e)] Concentration of ethanol in g L^{-1}. [f)] The yields of glucose, xylose, and ethanol were calculated based on the theoretical amounts, 463 mg, 249 mg, and 237 mg, respectively. [g)]In the case of the LMAA-pretreatment without washing with water.

51.6% and 30.8% yields, respectively (run 1). In the LMAA-pretreated napiergrass case, the yields of ethanol and xylose increased to 59.7% and 47.6% yields, respectively (run 2). However, the yields were still low. Therefore, we used a xylanase (Sumizyme X) in addition to the cellulase. The fraction of xylanase (F_X) in the mixture (133 mg) of cellulase and xylanase was defined. The maximum yields were observed at 0.4 of F_X among 0.1 - 0.7 of F_X (runs 3-9). The yields of ethanol and xylose reached 91.2% and 62.9%, respectively under the optimal condition of the SSF where the LMAA-pretreated napiergrass (1.33 g) was treated in a buffer solution (8 mL) using cellulase (80 mg), xylanase (53 mg), and the cell suspension (0.16 mL) of *S. cerevisiae* (run 6). On the other hand, in the non-treated napiergrass case, the ethanol yield was still low even at 0.4 of F_X (run 10). In the case of LMAA-pretreatment that was not washed with water (run 11), the ethanol yield was 75.1%. This showed that washing with water was needed to enhance the ethanol yield.

3.4. Pentose Fermentation with *E. coli* KO11

The SSF process was scaled up under optimized conditions of an acetate buffer (600 mL) containing cellulase (6.0 g), xylanase (4.0 g), the suspension of *S. cerevisiae* (12.0 mL), and the LMAA-pretreated napiergrass (100 g) was stirred at 34˚C. After the SSF for 18 h, 17.4 g (77.2%) of ethanol and 12.6 g (52.8%) of xylose were formed whereas 2.2 g (5.0%) of glucose remained unchanged. Ethanol was removed from the SSF-treated solution (600 mL) by distillation under a reduced pressure until the volume of the residual solution reached 500 mL. The condensed SSF solution contained 25.2 g/L of xylose and 4.4 g/L of glucose.

A portion (50-80 mL) of the inoculum culture *E. coli* KO11 was poured into the condensed SSF solution (40 - 50 mL). After pH was adjusted to 6.6, the PF was performed by shaking the solution at 150 rpm at 37˚C for 96 h. The yields of ethanol are summarized in **Table 3**. Ethanol (531 mg) was obtained under optimal conditions where the cell suspension of *E. coli* KO11 (70 mL) and SSF solution (40 mL) were used (run 4). Since the glucose disappeared after being fermented by *E. coli* KO11, it was assumed that the glucose was entirely turned to ethanol. Therefore, the ethanol amount produced from xylose was calculated to be 441 mg by subtracting 90 mg from 531 mg. Thus, the ethanol yield from xylose was determined to be 86.3%.

4. Discussion

Usual pretreatment of lignocelluloses has been performed by the removal of the lignin-component using an alkali-pretreatment. However, it was found that the alkali-pretreatment was not a useful method for the biological process of napiergrass with its low lignin-content, because of the retardation of the fermentation rate by the inhibitory materials derived from the alkali-pretreatment and the loss of nutrients for the fermentation [19]. The LMAA-pretreatment did not take place in the retardation of fermentation, as shown in **Figure 1** where the time profile of CO_2 evolution per 1 g of hollocellulose were compared among LMAA-pretreated, non-treated, and alkali-pretreated napiergrass. Moreover, it is possible to easily recycle gaseous ammonia with low energy in LMAA-pretreatment compared with the AFEX and SAA pretreatments which demanded energy to strip and recover ammonia from aqueous ammonia. Thus, the LMAA-pretreatment will be one of the useful pretreatment method that can be achieved with low energy to enhance the SSF processes of lignocelluloses.

5. Conclusion

A successfully efficient bio-ethanol production from napiergrass was achieved by the combination of the LMAA-pretreatment, the SSF used a mixture of cellulase and xylanase, and the pentose was fermented by *E. coli* KO11. The total mass balance was constructed in **Figure 2**. With 100 g of the LMAA-pretreated napiergrass, 24.1 g of ethanol can be totally produced through SSF and PF. The total ethanol yield reached 68.9% based on the amounts of xylan (21.4 g) and glucan (39.7 g) in

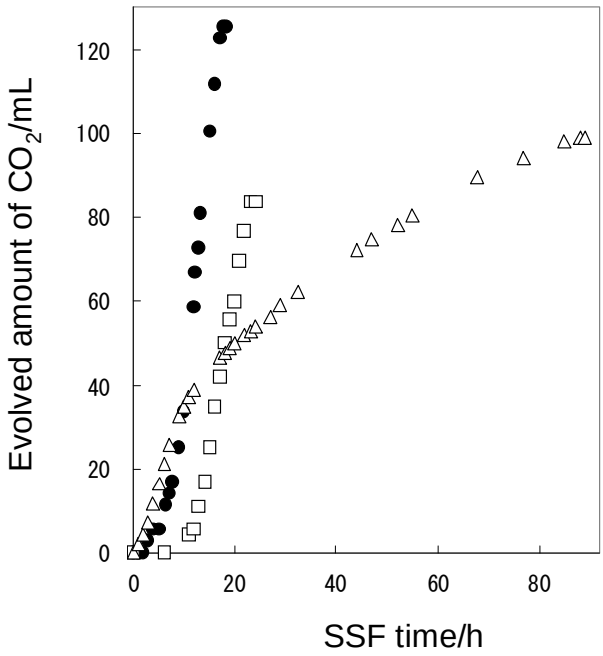

Figure 1. The time-conversion of CO_2 evolved from the SSF reaction of LMAA-treated (●), non-treated (□), and alkali-treated (△) napiergrass. The amounts of CO_2 were presented as the evolved CO_2 from 1.0 g of holocellolose.

Table 3. Pentose fermentation with *E. coli* KO11.

Run	KO11/mL[a)]	SSF/mL[b)]	Xylose/mg (Yield/%)[c)]	Ethanol/mg (Yield/%)[d)] [Yield/%][e)]	Ethanol/g·L^{-1}[f)]
1	50	50	807 ± 9 (64.0)	352 ± 28 (46.5) [37.2]	3.52
2	60	50	457 ± 122 (36.3)	440 ± 75 (58.1) [50.8]	4.00
3	70	30	0 (0.0)	309 ± 3 (72.0) [68.6]	3.09
4	**70**	**40**	**0 (0.0)**	**531 ± 4 (88.4) [86.3]**	**4.82**
5	70	50	60 ± 10 (4.7)	594 ± 77 (77.5) [73.6]	4.95
6	80	50	86 ± 38 (6.7)	540 ± 59 (70.4) [65.4]	4.15

[a)] The volume of the cell suspension of *E. coli* KO11 in ml. [b)]Pentose fermentation was performed for the SSF solution which contained 25.2 g·L^{-1} of xylose and 4.4 g·L^{-1} of glucose. [c)] Recovered xylose. [d)] The values in parenthesis are ethanol yields from both xylose and glucose. [e)] The values in blanket are the ethanol yields from xylose. [f)] Concentration of ethanol in g·L^{-1}.

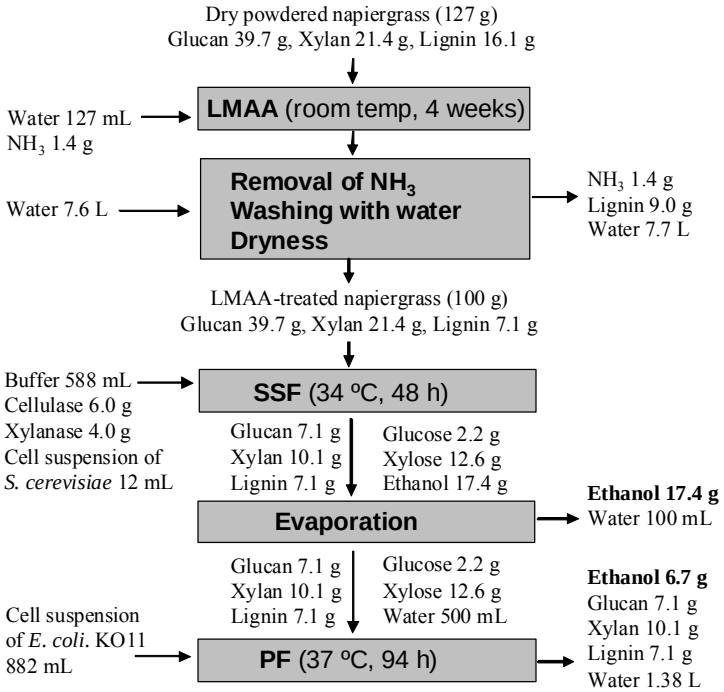

Figure 2. Process outline and total mass balance from LMAA-pretreated napiergrass (100 g).

100 g of LMAA-pretreated napiergrass. If the PF process was combined with the SSF process run 6 of **Table 2** which was run under optimal conditions of the small scale using 1.33 g of LMAA-pretreated napiergrass, the total yield will become 80.5%.

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