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Washing Lime-Pretreated Rice Straw with Carbonated Water Facilitates Calcium Removal and Sugar Recovery in Subsequent Enzymatic Saccharification

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Abstract: Generally, $Ca(OH)_2$ pretreatment of lignocellulosics for fermentable sugar recovery requires a subsequent washing step for calcium removal and pH control for optimized saccharification. However, washing $Ca(OH)_2$-pretreated feedstock with water is considered problematic because of the low solubility of $Ca(OH)_2$ and its adsorption to biomass. In this study, we estimated the availability of carbonated water for calcium removal from the slurry of $Ca(OH)_2$-pretreated rice straw (RS). We tested two kinds of countercurrent washing sequences, four washings exclusively with water (W4) and two washings with water and subsequent two washings with carbonated water (W2C2). The ratios of calcium removal from pretreatment slurry after washing were 64.2 % for the W4 process and 92.1 % for the W2C2 process. In the W2C2 process, 49 % of the initially added calcium was recovered as CaO by calcination. In enzymatic saccharification tests under a CO_2 atmosphere at 1.5 atm, in terms of recovery of both glucose and xylose, pretreated, feedstock washed through the W2C2 process surpassed that washed through the W4 process, which could be attributed to the pH difference during saccharification: 5.6 in the W2C2 process *versus* 6.3 in the W4 process. Additionally, under an unpressurized CO_2 atmosphere at 1 atm, the feedstock washed through the W2C2 process released 78.5 % of total glucose residues and 90.0 % of total xylose residues. Thus, efficient removal of calcium from pretreatment slurry would lead to not only the recovery of added calcium but also the proposal of a new, simple saccharification system to be used under an unpressurized CO_2 atmosphere condition.

Key words: Lime-pretreatment, rice straw, carbonated water, calcium recovery, enzymatic saccharification, CaCCO (Calcium Capturing by Carbonation) process

INTRODUCTION

The demand for efficient processes for chemical production from renewable resources is growing in today's worldwide trend of a sustainable economy with fewer adverse impacts on earth.[1] Herbaceous lignocellulosic biomass, such as rice straw (RS), wheat straw, and corn stover, is considered promising renewable feedstock for such processes because of its stable production from agriculture and low variation in qualities.[2] Lignocellulosics comprise two types of polysaccharides, cellulose and xylan, providing us, through their hydrolysis, with fermentable sugars, glucose and xylose, respectively, for further conversion to valuable chemicals. Many researchers focus on enzymatic hydrolysis for fermentable sugar production, and appropriate pretreatment of feedstock by chemical, physical, or biological means is a prerequisite for obtaining satisfactory sugar yields.[3)4]

Pretreatment of herbaceous feedstock with lime, or $Ca(OH)_2$ for efficient saccharification has gained considerable attention from many researchers because of the low price of $Ca(OH)_2$[5] and its ecological friendliness (e.g., in agriculture, dairy farming, and the food industry); sometimes, demonstration tests for $Ca(OH)_2$ pretreatment of biomass are performed in open facilities using simple equipment. However, $Ca(OH)_2$ pretreatment has one issue in the subsequent washing step: the removal of calcium from pretreatment slurry is difficult because of the low solubility of $Ca(OH)_2$ in water and its adsorption to biomass.[6] Also, using acids such as acetic acid and hydrochloric acid to release calcium into the solution, despite having a good effect on calcium removal, significantly raises the cost of the washing step and prevents calcium reuse in some cases.

In this study, we estimated the availability of carbonated water for calcium removal from $Ca(OH)_2$-pretreated RS slurry. The well-known phenomena of calcium ions forming water-insoluble calcium carbonate, $CaCO_3$, in carbonated water and further changing the structure to water-soluble calcium bicarbonate, $Ca(HCO_3)_2$, is the basis for the use of carbonated water to increase the solubility of calcium ions and their recovery in insoluble form. Chang *et al.* (1998) reported synthetic data on $Ca(OH)_2$ recovery from pretreatment slurry of crop residues and evaluated an appa-
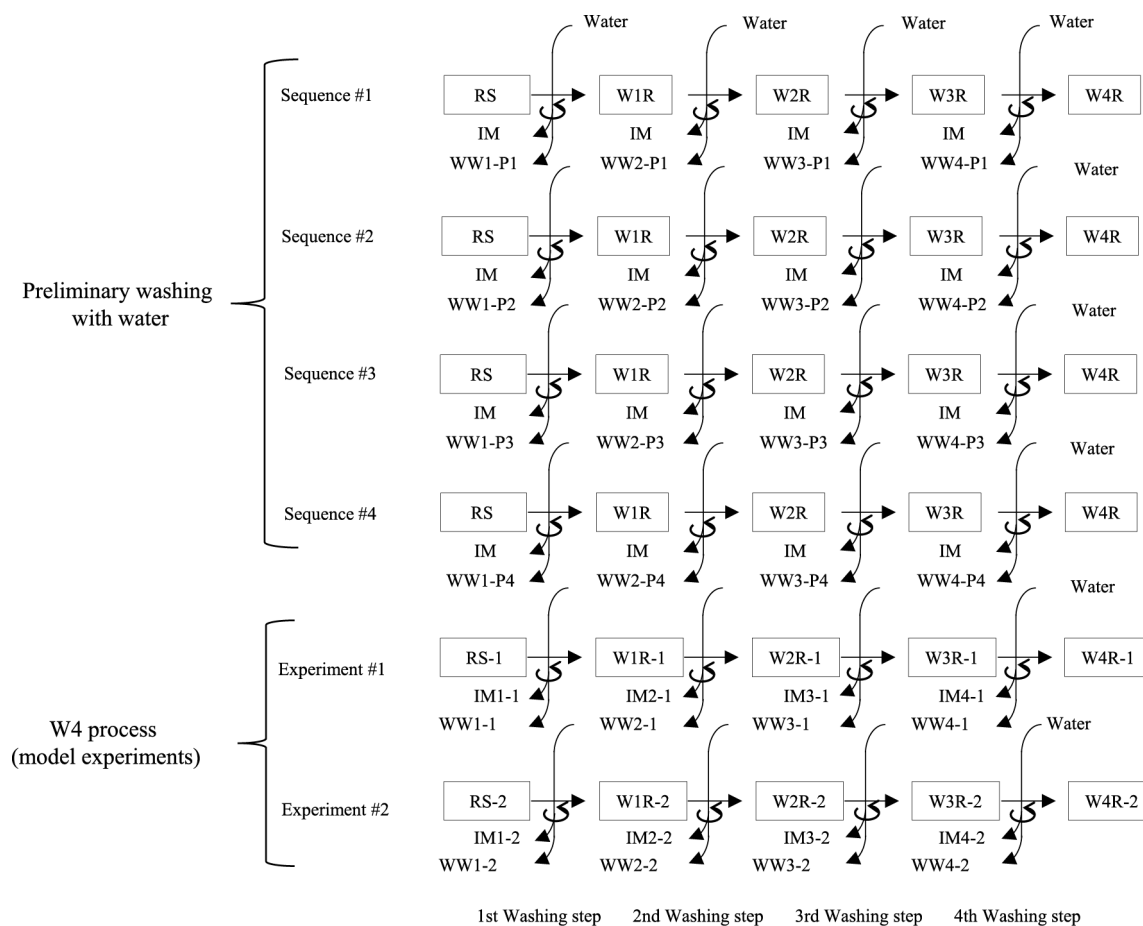
Fig. 1. The total flow of countercurrent washing system (W4).

RS, lime-pretreated RS; W*m*R, solid residue recovered at washing step *m*; WWm-P*n*, wash water recovered at washing step *m* of preliminary sequence *n*. After each washing step, the liquid fraction obtained after removal of solid residue was centrifuged to recover a precipitate (IM) and a supernatant. Two experiments (Experiment #1 and #2) were performed to calculate the average data. W*m*R-*n*, solid residue recovered at washing step *m* of experiment *n*; IM*m-n*, IM recovered as precipitate after centrifugation at washing step *m* of experiment *n*; WW*m-n*, wash water recovered as supernatant after centrifugation at washing step *m* of experiment *n*. Fractions of W4R-1 and W4R-2 were used for enzymatic saccharification.

ratus for continuous $Ca(OH)_2$ recovery using carbonated water.[7] In contrast, in this study, we tested two types of countercurrent washing sequences: four washings with water (W4) and two subsequent washings each, first with water and then with carbonated water (W2C2).

The methods of neutralization and further pH control under weak acidic conditions affect the recovery of fermentable sugars via enzymatic saccharification. Neutralization of $Ca(OH)_2$ with acetic acid after $Ca(OH)_2$ pretreatment significantly inhibits cellulase activity.[5] In laboratory-scale enzymatic saccharification experiments, either sodium acetate buffer or sodium citrate buffer is generally used. On the other hand, in larger-scale reactors, to save the cost of reagents, only pH adjustment with an acid or an alkali is used for saccharification. We developed the "Calcium Capturing by Carbonation" (CaCCO) process in which the pH of the slurry after $Ca(OH)_2$ pretreatment of feedstock is lowered by pressurization by CO_2 gas as acid for saccharification at a pH below 6.5.[8)9)10)11] Although the pressure of CO_2 needed for appropriate pH control depends on the amount of residual calcium in the slurry, the washing step could affect saccharification efficiency in CaCCO at a specific CO_2 pressure value. In this study, we also examined the possibility

of simplification of CaCCO by using pretreated, washed feedstock for saccharification in a CO_2 atmosphere.

MATERIALS AND METHODS

Materials. Sun-dried RS (Koshihikari, less than 10 % moisture content) was harvested from fields of the Central Region Agricultural Research Center, National Agriculture and Food Research Organization, Japan. The RS was cut into 2–3 cm pieces and stored at ambient temperature until use. β-Glucosidase (Novozyme 188) was purchased from Novozymes Japan Ltd. (Chiba, Japan), and industrial-grade CO_2 gas was purchased from Taiyo Nippon Sanso (Tokyo, Japan). The other chemical reagents were of analytical grade.

$Ca(OH)_2$ pretreatment. RS (corresponding to 1.5 kg dry matter [DM]) was mixed with tap water (1,650 mL) and $Ca(OH)_2$ (0.15 kg) and then milled with a wet-milling machine (Shokusenki, Shinko Engineering, Co., Ltd., Kobe, Japan). The milled RS was packed in an airtight plastic bag to prevent contact with CO_2 in air and settled in a dark room at 25 °C for 30 days.

Washing sequence with water. Countercurrent washing
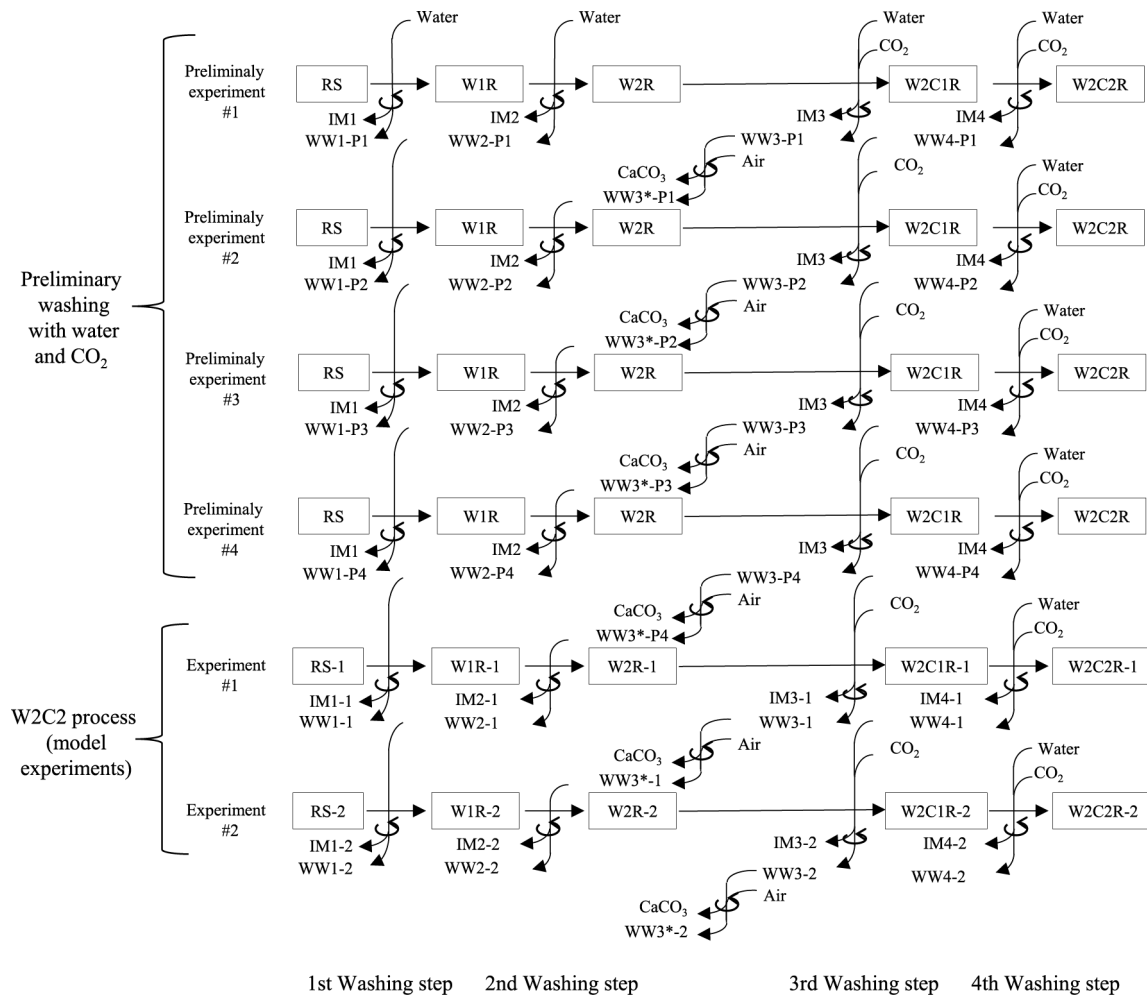
Fig. 2.   Overall illustration of water/carbonated water washing of pretreated RS for the W2C2 process.
    The numbering system of washing residues, IMs, and wash water are similar to that of Fig. 1. W2C1R and W2C2R indicate the residues washed with carbonated water. Aeration was performed for each WW3 fraction, and each fraction was centrifuged to separate $CaCO_3$ as a precipitate and the WW3* fraction as a supernatant. W2C2R-1 and W2C2R-2 were used for enzymatic saccharification, and $CaCO_3$ recovered from WW3-1 and WW3-2 were used for calcination to regenerate CaO. WW1-1 and WW1-2 were also used to precipitate $CaCO_3$ by adding ammonium water (see Fig. 6).

was used to save the amount of wash water. Figure 1 summarizes the total flow of countercurrent washing system (W4). The preliminary washing sequence was performed four times to equilibrate the countercurrent washing system. In other words, preliminary washing sequences were required at least three times to prepare WW2-P4 (originated from water added in preliminary washing #2). In the first preliminary washing sequence, 1 L distilled water was added to pretreated RS (47 g of DM), with constant mixing with a stirring blade for 30 min in the container. The washed solid fraction was trapped on three-layered gauze and manually squeezed; we referred to the squeezed solid fraction (water content = approximately 60–70 %) as "W1R." Next, we added fresh water to W1R to obtain W2R. This step was repeated four times, and the wash water obtained each time was centrifuged at 8,000 × G for 10 min to separate the insoluble material (IM) from the supernatant. IMs were slurry-like particles passing through the three-layered gauze used for squeezing. Each washing solution (WW2-P1, WW3-P1, and WW4-P1) was reused to wash the pretreated RS in the second preliminary washing sequence; that is, we reused the WW2-P1 solution produced after

washing W1R in the first preliminary washing sequence (#1) to wash pretreated RS in the second sequence (#2). In each washing sequence, 1 L fresh distilled water was used to wash W3R. The solutions used for washing pretreated RS were discharged from the countercurrent system as final wastewater (WW1-P1, P2, P3, P4). The WW2-P4, WW3-P4, and WW4-P4 solutions were used to wash pretreated RS (RS-1) or washed solid fractions (W1R-1, W2R-1, and W3R-1) to obtain experimental data in the W4 process. In the experiment #1, wash water obtained each time was centrifuged at 8,000 × G for 10 min to separate IMs (IM1, 2, 3, and 4-1) from the supernatant. The supernatants (WW2-1, 3-1, 4-1) were used to wash the solids in the subsequent experiment #2. The weight and chemical composition of samples obtained in experiments #1 and #2 were analyzed to evaluate the process.

Washing sequence with water and carbonated water (W2C2). To enhance the elution of calcium from the washed solid fraction, the washing sequences in the W4 process (Fig. 1) were modified using carbonated water in the third and fourth washing steps in each washing sequence; Fig. 2 show the experiments performed. The scheme of the first

and second washing steps using water was the same as that shown in Fig. 1. However, the third and fourth steps were modified to enhance Ca solubilization by using CO_2. CO_2 gas was supplied from the bottom of container at a flow rate of 60 mL/min, with continuous shaking using a stirring blade for 30 min. The wash water obtained each time was centrifuged at $8,000 \times G$ for 10 min to separate the IM from the supernatant (WW1, 2, 3, and 4 - P1). Calcium in WW3-P1 was recovered as a precipitate (mainly $CaCO_3$) by aeration for 9 h and subsequent centrifugation at $8,000 \times G$ for 10 min. The supernatant is shown as WW3*-P1. The WW2-P1, WW3*-P1, and WW4-P1 fractions were used to wash pretreated RS (47 g of DM) or washed solid fractions (W1R, W2R, W2C1R) in the second preliminary experiment (#2). After the 4th preliminary experiment (#4), the model experiments (#1 and #2) were performed to obtain experimental data in the W2C2 process. Aliquots of samples obtained in experiments #1 and #2 in Fig. 2 were taken and used to evaluate the process.

Calcium regeneration. Calcium in the first wash water in the W2C2 process (WW1-1 or WW1-2; Fig. 2) was recovered as follows:

(i) CO_2 gas was introduced (60 mL/min) in the wash water for 5 min, and ammonia water (5.3 M) was added (final 150 mM) to precipitate calcium ions as $CaCO_3$.

(ii)   The precipitate was recovered by centrifugation at $8,000 \times G$ for 10 min.

(iii)   Calcium recovery from WW3-1 and WW3-2 (Fig. 2) was performed by aeration, as described in the "***Washing sequence with water and carbonated water (W2C2)***" section.

(iv)   Precipitates from the first and third wash water were dried, weighed, and crushed with a ball-milling machine (BM-301, Verder Scientific Co., Ltd., Tokyo, Japan).

(v)   The crushed powder (50 mg) was burned in a melting pot to remove any organic materials, mixed with 100 mM HCl (20 mL), and the mixture was centrifuged ($8,000 \times G$ for 10 min) to obtain a supernatant.

(vi)   The calcium concentration in the supernatant was measured by chelating it with chlorophosphonazo-III (metallo assay calcium test, Funakoshi, Tokyo, Japan). The powder was also calcined at 900 °C for 90 min in a muffle furnace to convert $CaCO_3$ to CaO.

(vii)   The CaO content in the calcined powder was evaluated by titration with 200 mM ammonium acetate in dehydrated ethylene glycol at 80 °C according to the official method (JCASI-01:1997, Japan Cement Association Standard).

Enzymatic saccharification. Enzymatic saccharification experiments were performed with some modifications, as described previously.[10] Aliquots (equivalent to 1.5 g of DM) of solid fractions (pretreated RS: W4R-1 and W4R-2 in Fig. 1; W2C2R-1 and W2C2R-2 in Fig. 2) were put in airtight vials. CO_2 gas was introduced into the vials at 1.5 atm and 4 °C for 12 h. This step was repeated one more time, and the vials were divided into three groups. In group 1, the samples were neutralized with 5 M HCl, and then sodium acetate buffer (final 50 mM) was added to adjust the pH at 4.4–4.8. In groups 2 and 3, CO_2 gas was introduced

into the void spaces in the vials; the proportion of void space to slurry was 4:1. A cellulase-containing culture medium of *Trichoderma reesei* M2-1 strain[12] (6.9 FPU/g of DM) and β-glucosidase (Novozyme 188, 60.7 CbU/g of DM, Novozymes) were used as enzymes. Sodium azide (final concentration 0.02 %) and chloramphenicol (final concentration 50 μg/mL) were added to prevent microbial contamination. The total solution volume in all the vials was adjusted with distilled water to 13.5 mL. In groups 2 and 3, the void spaces in the vials were replaced with unpressurized CO_2 and pressurized CO_2 (1.5 atm), respectively. Enzymatic saccharification was performed at 50 °C and 150 rpm. CO_2 gas in group 2 and 3 vials was refilled after 24 h of saccharification. After 48 h of saccharification, the pH values in the vials were measured as soon as the caps were opened. The amounts of liberated monomeric sugars (glucose and xylose) and oligomeric sugars (glucose and xylose oligomers) were determined as follows: The hydrolysates (0.12 mL) were mixed with sulfuric acid (final conc. was 9 %) and heated at 100 °C for 2 h. After centrifugation ($13,000 \times G$ for 5 min), the supernatants were neutralized by equal volume of $CaCO_3$ slurry (1 g : 3 mL H_2O) . After centrifugation ($13,000 \times G$ for 5 min), the supernatants were analyzed for glucose and xylose measurements. The sugar standard solution (10 mg/mL glucose, 4.14 mg/mL xylose) was treated with 9 % H_2SO_4 in parallel with the samples to estimate the monomeric sugar degradation rate between the heating step.

Component analysis. Dried samples of pretreated RS or washed RS were ball-milled for component analysis. The total glucan (TG), total xylan (TX), Klason lignin (KL), acid-soluble lignin (ASL), and ash contents were analyzed, as described previously.[10] DM was defined as "solid materials after drying the sample at 105 °C for 18 h." The involatile material content in liquid samples was estimated by drying the samples (10 mL) in aluminum dishes at 105 °C and then measuring the weight of the residues.

RESULTS AND DISCUSSION

Calcium removal from pretreated RS.

In this study, we investigated two countercurrent washing sequences, W4 and W2C2, for washing out calcium as well as other solubilized materials from $Ca(OH)_2$-pretreated RS (Figs. 1 and 2). After four washings of $Ca(OH)_2$-pretreated RS in the W4 process, the W4R fraction still contained 35.8 % of the initially added calcium ($Ca(OH)_2$ pretreatment), whereas the W2C2R fraction in the W2C2 process contained only 7.9 % of calcium (Table 1). In the W4 process, approximately half of the calcium was washed out in the first washing step. However, calcium removal during the second, third, and fourth washing steps was significantly inefficient, which could be attributed to both low solubility of $Ca(OH)_2$ in water and the interaction of calcium with lignin and xylan at high pH values.[13] IMs also contained substantial amount of calcium (Table 1). The calcium contents of IMs were higher than that of pretreated RS or washed RS. The small particles of insoluble $Ca(OH)_2$ or $CaCO_3$ might pass through the gauze mesh during the

squeezing processes. Table 2 shows the calcium content in liquid fractions in both W4 and W2C2 washing processes (0.9 g; approximately 19 % of Ca contained in pretreated RS).

The WW1-1/WW1-2 fraction contained calcium at a high concentration of approximately 20 mM in both W4 and W2C2 processes (Table 2). $Ca(OH)_2$ pretreatment of RS cleaves ester linkages in its cell walls and generates organic acids, such as acetic acid, ferulic acid, and *p*-coumaric acid,[14)15)] which would raise the solubility of calcium ions by reactions with $Ca(OH)_2$. A significant difference was observed in the calcium content in the WW3-1/WW3-2 fraction in the W4 process (3.3 mM) and correspondingly in the W2C2 process (21.6 mM). In the W2C2 process, carbonated water was used at the third and fourth steps for washing the corresponding solid fractions, in which calcium existed in its more water-soluble form, $Ca(HCO_3)_2$, compared to the less soluble forms $Ca(OH)_2$ and $CaCO_3$. The time course of solubilization of calcium in carbonated water from the W2R-1/W2R-2 fraction (open circles) and the W2C1R-1/W2C1R-2 fraction (solid circles) in the W2C2 process are indicated in Fig. 3. Under the conditions tested, bubbling of CO_2 for 30 min seemed sufficient for maximum elution of calcium into the solution, and the pH became stable around 6.6 in the W2R-1/W2R-2 fraction and 6.0 in the W2C1R-1/W2C1R-2 fraction. After calcium solubilization, air was introduced into the WW3-1/WW3-2 solution for 9 h to purge the dissolved CO_2 without adding any chemical reagents. During this process, the precipitants appeared gradually, and the calcium concentration decreased from 21.6 to 4.4 mM (Fig. 4 and Table 2), suggesting that removal of dissolved CO_2 by aeration converts $Ca(HCO_3)_2$ in solution to the much less soluble $CaCO_3$. Chang *et al.* investigated the separation of calcium from $Ca(OH)_2$-pretreated lignocellulosics by washing pretreated feedstock with water (either batch or continuous method), similar to our W4 process.[7)] The authors emphasized the importance of pH control to 9.5 to efficiently precipitate calcium as $CaCO_3$, and they proposed washing feedstock 6 to 10 times in the batch method. Our W2C2 process is distinct from theirs in terms of more acidic washing conditions to efficiently dissolve calcium as $Ca(HCO_3)_2$.

Table 3 shows the recovery and components of pretreated RS after washing. W4R remained as 65.6 % of glucan and 68.3 % xylan of the $Ca(OH)_2$-pretreated RS before washing, whereas W2C2R remained as 72.0 % glucan and 71.0 % of xylan. The significant loss of fermentable sugars in both samples was mainly attributed to the loss in the initial washing step. For example, in the W4 process, 18.9 % of glucan and 14.8 % of xylan in the pretreated RS were rinsed out in the initial washing step (data not shown). Karr and Holtzapple reported that washing $Ca(OH)_2$-pretreated corn stover results in 10 % loss of total sugars,[16)] whereas in our study, the loss in the initial washing step was more significant. We speculated that differences in structures of sugars and/or fibers between RS and corn stover might cause this discrepancy. The average glucan and xylan contents of IMs were 16.9 and 5.1 %, respectively. Because the total DM of IMs was relatively less, the loss of glucan and

Table 1. Dry matter and calcium contents of solid fractions before and after washing of lime-pretreated rice straw (RS).

Washing process	Solid fraction	Dry matter (g)	Calcium content (%) / total weight (g)	Residual calcium (%)
	Pretreated RS	100	4.7 / 4.7	100
W4 process	W1R	66.4	3.8 / 2.5	54.2
	W2R	60.4	3.5 / 2.1	45.6
	W3R	57.6	3.4 / 1.9	42.2
	W4R	54.6	3.1 / 1.7	35.8
	IMs(1-4)	12.7	6.9 / 0.9	-
W2C2 process	W1R	72.7	3.8 / 2.8	58.2
	W2R	66.1	3.4 / 2.2	48.0
	W2C1R	58.2	1.2 / 0.7	15.4
	W2C2R	53.8	0.7 / 0.4	7.9
	IMs(1-4)	12.0	7.7 / 0.9	-

Details of two washing processes as well as the definition of solid fractions are shown in Figs. 1 and 2. "Calcium content (%)" and "Residual calcium (%)" are defined as (the weight of calcium (g)/the weight of dry matter (DM, g))×100 (%) and (the weight of calcium in solid fraction (g)) / the weight of calcium in pretreated RS (g) ×100 (%), respectively. The average value of two experiments (Experiments #1 and #2, Figs. 1 and 2) are shown. All values in this table are shown after recalculation under the assumption that 100 g DM was used as starting material for washing for the convenience of comparison. IMs(1-4) indicates the total weight of DM (g), average Ca content (%), total weight of Ca (g) of the centrifuged precipitates of washing solutions (WW1 - WW4), respectively.

Table 2. Volumes, calcium- and acetate concentration of liquid fractions generated during washing processes for lime-pretreated rice straw (RS).

Washing process	Liquid fraction	Volume (L)	Calcium conc. (mM)	Acetate conc. (mM)
W4 process	WW1-1/WW1-2	2.01	22.7	12.9
	WW2-1/WW2-2	1.97	4.6	0.5
	WW3-1/WW3-2	1.95	3.3	n.d.
	WW4-1/WW4-2	1.96	2.4	n.d.
W2C2 process	WW1-1/WW1-2	2.10	19.7	12.9
	WW2-1/WW2-2	2.08	6.0	0.5
	WW3-1/WW3-2	2.07	21.6	n.d.
	WW3*-1/WW3*-2	2.07	4.4	n.d.
	WW4-1/WW4-2	2.06	3.5	n.d.

Details of two washing processes as well as the definition of liquid fractions are shown in Figs. 1 and 2. The average values of two experiments (Experiments #1 and #2, in Figs. 1 and 2) are shown. All values in this table are shown after recalculation under the assumption that 100 g DM was used as starting material for washing for the convenience of comparison. n.d.: not detected.

xylan through IMs was negligible. After washing, KL and ASL significantly decreased, which is expected to have a positive effect on enzymatic saccharification as lignin might have inhibitory effects on cellulose and xylan saccharification.[14)]

Calcium recovery from liquid fractions.

A decrease in the calcium concentration of the WW3 fraction in the W2C2 process by aeration is shown in Fig. 4

Table 3. Major chemical components in unwashed and washed solid samples of lime-pretreated rice straw (RS).

Washing process /solid fraction	Dry matter (g)	Calcium content (%)	Glucan content (%) / Glucan recovery (%)	Xylan content (%) / Xylan recovery (%)	Klason lignin content (%)	ASL content (%)	Ash content (%)
/Pretreated RS	100	4.7	30.7 / 100.0	12.8 / 100.0	10.9	2.1	24.6
W4 process /W4R	54.6	3.1	36.9 / 65.6	16.0 / 68.3	11.9	1.4	20.3
W2C2 process /W2C2R	53.8	0.7	41.1 / 72.0	16.9 / 71.0	11.8	1.3	17.0

Details of two washing processes as well as the definition of solid fractions are shown in Figs. 1 and 2. The average values of two trials (Experiments #1 and #2 in Figs. 1 and 2) are plotted. All values in this table are shown after recalculation under the assumption that 100 g DM was used as starting material for washing for the convenience of comparison. Recoveries of glucan and xylan are defined as (the weight of glucan in solid fraction (g)) / (the weight of glucan in pretreated RS (30.7 g) × 100 (%)) and (the weight of xylan in solid fraction (g)) / (the weight of xylan in pretreated RS (12.8 g)) × 100 (%) , respectively. ASL: acid soluble lignin.
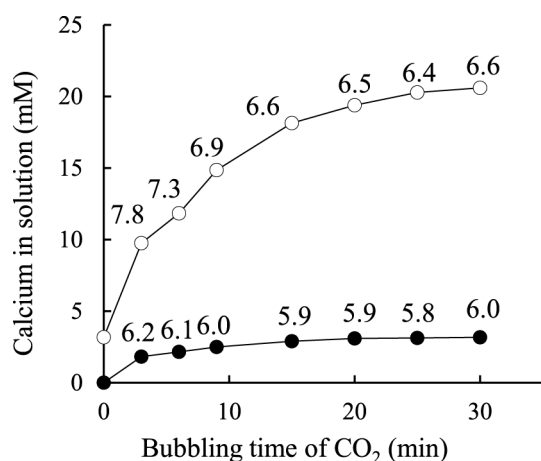
Fig. 3. Calcium solubilization from W2R and W2C1R in the W2C2 process via CO_2 introduction.
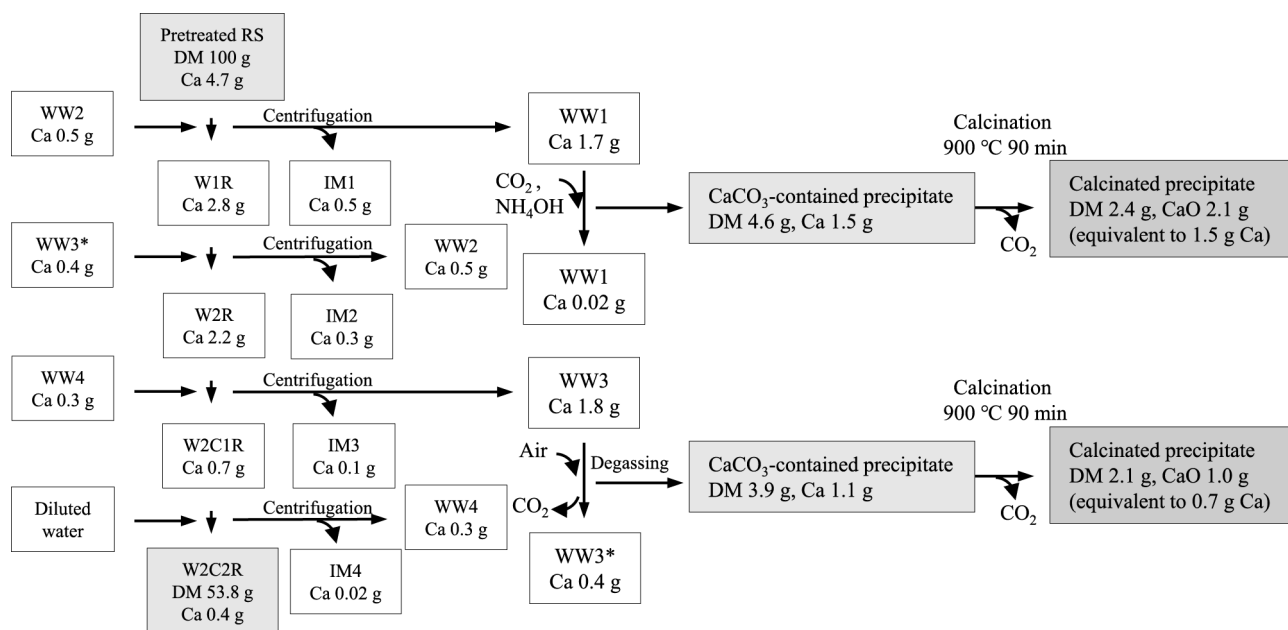
Details of the W2C2 process as well as the definition of liquid fractions are shown in Fig. 2. White and black dots indicate the data with W2R and W2C1R, respectively. The average values of two experiments (#1 and #2 in Fig. 2) are plotted. The number near each dot indicates the average pH value in the liquid fraction during bubbling.
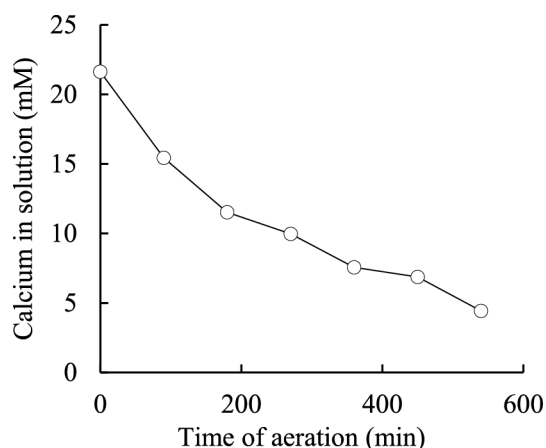
Fig. 4. Calcium precipitation in the WW3-1/WW3-2 fraction in the W2C2 process by aeration.

Details of the W2C2 process as well as the definition of liquid fractions are shown in Fig. 2. The average values of two experiments (#1 and #2 in Fig. 2) are plotted.

(discussed before). Although this step was time consuming, the calcium concentration in solution decreased without the use of alkalis or heat treatment. The decrease in calcium concentration (21.6 to 4.4 mM) indicated that approximately 1.4 g calcium in 2.1 L solution was precipitated

(Fig. 5). The precipitate (DM 3.9 g) was solubilized with 100 mM HCl to estimate the calcium content. It resulted in the total weight of calcium solubilized with HCl to be 1.1 g, approximately 79 % of calcium precipitated in the WW3 fraction (1.4 g). The reason for the discrepancy of calcium recovery was unknown. Next, we performed CaO regeneration from $CaCO_3$ by calcination of the precipitate at 900 °C for 90 min. The estimation of CaO contents after calcination indicated that 0.7 g calcium [64 % of the calcium in the precipitate (1.1 g)] was converted into calcium oxide. The calcium recovery rate from WW3 fraction needs to be improved in the future. To recover calcium from the WW1 fraction, we used the ammonia-aided precipitation method given by Chang *et al.*[7] Initially, CO_2 gas was introduced into the fraction for 5 min, which gave us no precipitate of $CaCO_3$ (Fig. 6). In a pH range between 7.8 and 8.5, we observed a dramatic decrease in calcium solubility, and a further increase in pH was effective in thoroughly precipitating calcium into the solution. Finally, almost all the calcium was precipitated at pH 9.3. These results indicated that mere introduction of CO_2 gas has no positive effects on calcium precipitation but a pH shift to around 8 is critical for the formation of $CaCO_3$ in the presence of organic acids such as acetic acid. This ammonia-aided calcium precipitation method could be adopted for the WW3 fraction for much better recovery of calcium compared with the aeration system, whereas the cost of ammonia would significantly affect the total cost of calcium recovery. The precipitates derived from the WW1 fraction (DM 4.6 g) were solubilized with 100 mM HCl to estimate the calcium content. It resulted in the total weight of calcium solubilized with HCl to be 1.5 g, approximately 88 % calcium in the WW1 fraction (1.7 g). The reason of Ca loss (12 %) remains unknown; however, the discrepancy was relatively lower than that of WW3 (21 %). Since rice straw contains significant amount of silica comparing with another fibrous plants, we have predicted that the reaction between calcium and silica might affect the calcium recovery rate. The effects of silica to the calcium recovery process are under analysis at present. Next, the precipitate derived from the WW1 was also calcinated (Fig. 5). Alternative to the case of WW3, the calcium in the precipitate from the WW1 fraction was stoichiometrically converted to CaO by calcination. The difference in the CaO conversion rate between WW1 and WW3 might be the clue to speculate the factors that inhibit

Fig. 5.   Regeneration of CaO from calcium in the WW1 and WW3 fractions.

All the values in the figure were converted to 100 g of DM as start material for the convenience of calculation. The weight of Ca indicated that of atomic form, except for CaO presented in the "calcinated deposits" derived from WW1 and WW3.
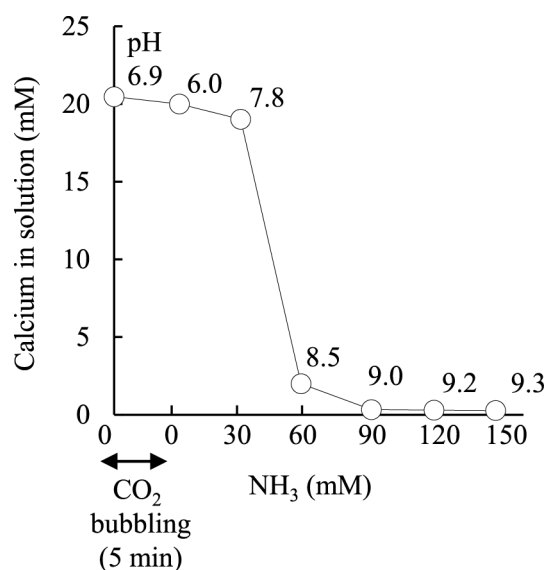
Fig. 6.   Calcium precipitation in the WW1-1/WW2-1 fraction in the W2C2 process by pH shift with ammonia.

Details of the W2C2 process as well as the definition of liquid fractions are shown in Fig. 4. The average value of two experiments (#1 and #2 in Fig. 2) are plotted. The number near each dot indicates the average pH value in the liquid fraction at each ammonia concentration.

the conversion of $CaCO_3$ contained in the latter precipitate. Overall, the recovery rate of CaO (equivalent of 1.5 g + 0.7 g Ca) from the $Ca(OH)_2$ consumed during RS pretreatment (equivalent of 4.7 g Ca) was approximately 49 %.

Enzymatic saccharification under CO_2 atmosphere.

In this study, we estimated the liberation of glucose and xylose in the reaction solution with pretreated, washed RS after 48 h of enzymatic saccharification under a defined reaction condition: (i) neutralization of slurry with HCl and subsequent pH control with sodium acetate at pH 4.5, (ii)

CO_2 pressurization at 1.5 atm, or (iii) an unpressurized CO_2 atmosphere at 1 atm (Fig. 7). Recovery of monomeric sugars from pretreated RS in the HCl–sodium acetate reaction condition was highest among the three reaction conditions (Figs. 7A and 7C). It is well known that most fungal cellulolytic and xylanolytic enzymes show optimum activity at a pH range between 4.5 to 5.0. Therefore, it seems that the each sugar recovery rate seems to reflect the gap between optimum pH for the enzymes and actual pH in the reaction solution. Table 3 shows how the amount of calcium in the reaction solution could significantly affect the pH condition. The W2C2R fraction contains the least amount of calcium, whereas the calcium content in the W4R fraction is more than that in the W2C2R fraction but still less than that in pretreated RS. The phenomenon is more significant in monomeric sugar liberation (Figs. 7A and 7C) than in monomeric + oligomeric sugar liberation (Figs. 7B and 7D), implying that monomeric-sugar-liberating enzymes (*i.e.*, β-glucosidases and β-xylosidases) are unstable or inactive at high pH. Another possibility could be the direct inhibitory effect of calcium ions on enzymatic saccharification.[17] Other components of the slurry of pretreated RS, such as lignin and xylose oligomers, which are removed during washing, may also affect saccharification efficiency.

CO_2 works as an acid to interact with calcium ions and generate water-insoluble $CaCO_3$, which reduces the inhibitory effect of calcium ions on enzymatic activities. In this study, in addition to sodium acetate buffer, we used CO_2 gas for conditioning the pH of reaction solutions. Pressurization at 1.5 atm of the W4R and W2C2R fractions during saccharification resulted in > 70 % recovery of glucose residues (monomers and oligomers; Fig. 7B) and > 80 % recovery of xylose residues (monomers and oligomers; Fig. 7D). On the other hand, in pretreated RS, approximately 50 % recovery of glucose residues and < 70 % recovery of
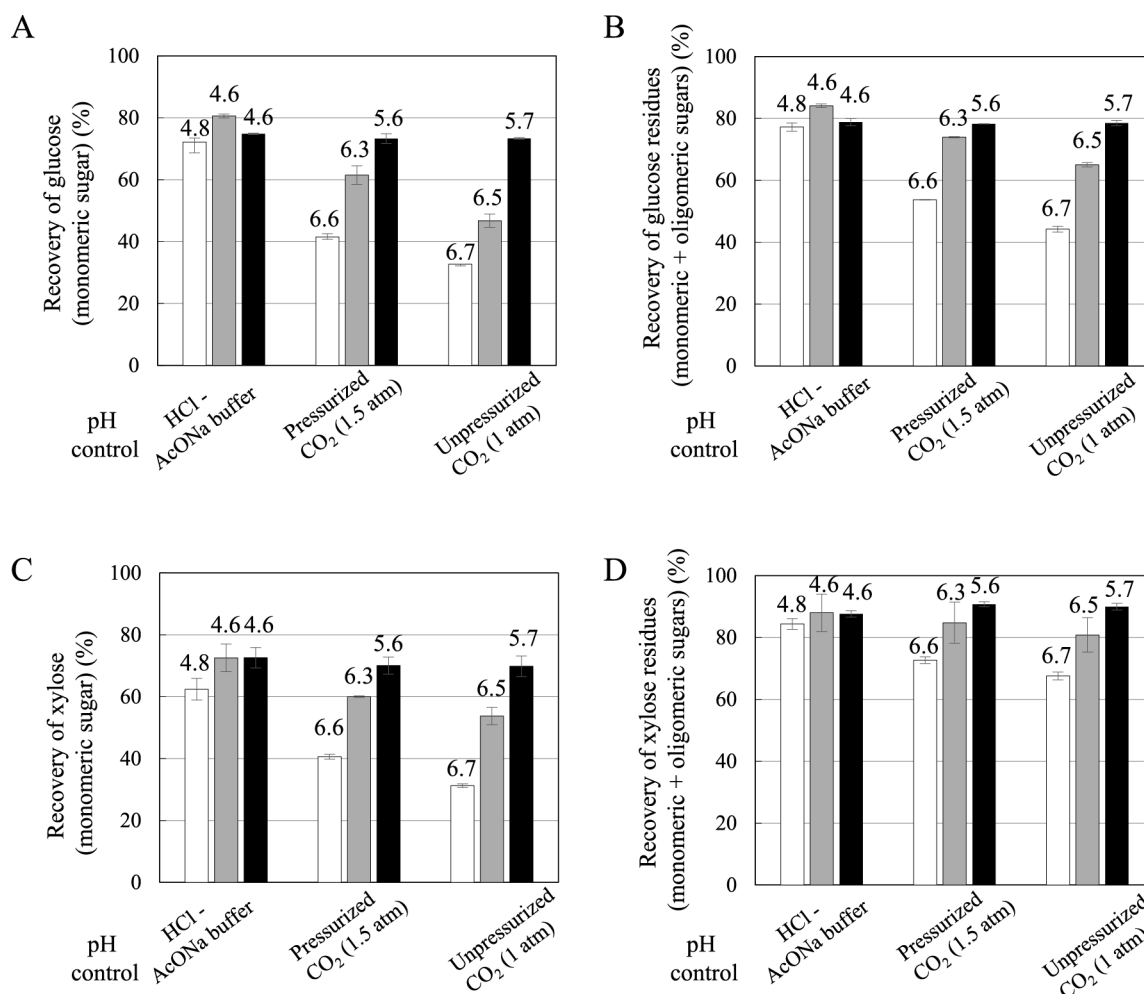
Fig. 7. Recovery of sugars in solution after 48 h of enzymatic saccharification of pretreated, washed RS.

Panel (A) and (B) indicate the recovery rate of glucose and xylose monomer, respectively. Panel (C) and (D) indicate the recovery rate of glucose and xylose (monomer + oligomers). The amounts of oligomers were measured as described in "Materials and Methods." Three kinds of substrates used for saccharification: $Ca(OH)_2$-pretreated RS (white bars), W4R fraction (gray bars), and W2C2R fraction (black bars) (see Table 3). Three conditions for pH control: (i) neutralization of slurry with HCl and subsequent pH control with sodium acetate at pH 4.5, (ii) CO_2 pressurization at 1.5 atm, and (iii) an unpressurized CO_2 atmosphere at 1 atm. Sugar recovery (%) is defined as follows: (Moles of glucose or xylose residues liberated in solution) / (Moles of glucose or xylose residues in substrate) ~ 100. The average values of two experiments (#1 and #2 in Figs. 1 and 2) with standard deviation are plotted. The number near each bar indicates the average pH value in the reaction solution during enzymatic saccharification.

xylose residues suggested that the amount of calcium ions in the slurry are too large to reduce the pH by CO_2 pressurization at 1.5 atm. Further pressurization with CO_2 would reduce the reaction pH, leading to better sugar recovery ratios.[11]

Next, we used an unpressurized CO_2 atmosphere at 1 atm during enzymatic saccharification of reaction slurries. We found that sugar recovery in the W2C2R fraction in unpressurized CO_2 conditions was as high as that in samples neutralized with HCl and sodium acetate. It is noteworthy that the pH of the reaction solution with the W2C2R fraction was 5.7, whereas the pH values with the other two fractions were > 6.5. Thus, instead of using acidic reagents for neutralization, thorough washing of biomass with water and carbonated water in the W2C2 process is effective for sugar recovery under mild conditions with an unpressurized CO_2 atmosphere. Another important advantage of mild conditions is the simple design of the reaction vessel for saccharification, without the need for a pressure-proof vessel.

In 2010, we developed CaCCO for sugar recovery from RS in which a slurry with $Ca(OH)_2$-pretreated RS is directly neutralized by CO_2 pressurization for enzymatic saccharification and fermentation.[9] The process skips a washing step of $Ca(OH)_2$-pretreated feedstock for sugar recovery for saccharification; RS may contain significant amounts of readily recoverable sugars, such as sucrose and starch, which are rinsed out in the wash water, in addition to alkali-solubilized xylan and small particles of fiber. In this study, we proposed simple and efficient saccharification in the W2C2R fraction, which could be a trade-off with high sugar recovery from feedstock in CaCCO. In 2013, we modified CaCCO by installing a simple washing step of $Ca(OH)_2$-pretreated feedstock to recover solubilized phenolic acid as a by-product.[14]

The W2C2 process in this study can be further modified for recovery of valuable materials from wash water. The simple, efficient saccharification in an unpressurized CO_2 atmosphere as well as simple pretreatment at ambient tem-

perature would significantly contribute to increasing the options for flexible process development, taking feedstock quality, size of the facility for conversion, and the wastewater management strategy into consideration.

Conclusion.

Countercurrent washing of $Ca(OH)_2$-pretreated RS with water and carbonated water in the W2C2 process enables us to adopt a new and simple saccharification process in an unpressurized CO_2 atmosphere for efficient recovery of fermentable sugars. The simplicity of calcium removal, recovery, and reactivation as CaO in the washing step should raise the feasibility of biomass conversion by lowering the stress on wastewater management and calcium/by-product recovery. Thus, upgrading the sugar platform for RS by process simplification could provide us with new opportunities for flexible production of biofuels and/or chemicals in rural areas in Japan and other countries.

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