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Note

Control of pH by CO_2 Pressurization for Enzymatic Saccharification of $Ca(OH)_2$-Pretreated Rice Straw in the Presence of $CaCO_3$

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Abstract: The aim of this study was to investigate the effect of pH control by CO_2 pressurization on the enzymatic hydrolysis of herbaceous feedstock in the calcium capturing by carbonation (CaCCO) process for fermentable sugar production. The pH of the slurry of 5 % (w/w) $Ca(OH)_2$-pretreated/CO_2-neutralized rice straw could be controlled between 5.70 and 6.38 at 50 °C by changing the CO_2 partial pressure (pCO_2) from 0.1 to 1.0 MPa. A mixture of fungal enzyme preparations, namely, *Trichoderma reesei* cellulases/hemicellulases and *Aspergillus niger* β-glucosidase, indicated that pH 5.5–6.0 is optimal for solubilizing sugars from $Ca(OH)_2$-pretreated rice straw. Enzymatic saccharification of pretreated rice straw under various pCO_2 conditions revealed that the highest soluble sugar yields were obtained at pCO_2 0.4 MPa and over, which is consistent with the expected pH at the pCO_2 without enzymes and demonstrates the effectiveness of pH control by CO_2 pressurization.

Key words: calcium capturing by carbonation (CaCCO) process, CO_2-pressurized enzymatic saccharification, fungal cellulase, fungal β-glucosidase

Lignocellulosic biomass, the most abundant renewable resource on earth, is considered as an alternative material to edible feedstocks for producing fermentable sugars, which can be further converted into value-added products including biofuels, bioplastics, and biochemicals.[1)2)3)] Lignocellulosic biomass mainly contains two structural polysaccharides, cellulose and xylan, as sources of fermentable sugars such as glucose and xylose. Generally, pretreatment steps that break down the rigid structure of the lignocellulosic matrix are required to enhance the accessibility of polysaccharide-hydrolyzing enzymes to the substrates. Pretreatment methods can be categorized as biological, chemical, physical, or physiochemical approaches.[4)5)6)] Alkali pretreatment is one of the chemical pretreatments that readily remove lignin and xylan side chains by the cleavage of ester bonds, resulting in improved efficiency of the subsequent enzymatic hydrolysis.

We developed a $Ca(OH)_2$-based alkali-pretreatment process called the "calcium capturing by carbonation (CO_2)" (CaCCO) process for sugar production from herbaceous feedstocks.[7)8)] In this process, after the alkali pretreatment step, $Ca(OH)_2$ is neutralized by carbonation to precipitate Ca ion as $CaCO_3$, which remains in the vessel during the subsequent enzymatic hydrolysis, and thus the solid–liquid separation step for washing the pretreated feedstock can be omitted. In a kg-scale conversion test of the CaCCO process, we adopted the enzymatic saccharification step under CO_2-pressurized conditions at a partial pressure of CO_2 (pCO_2) of 0.9 MPa in order to lower the pH of the $CaCO_3$-containing slurry, resulting in the successful recovery of dense sugar solution.[9)] It is realized that the solubilization of CO_2 by the pressurization would cause re-equilibration of the carbonate system by increasing the concentration of bicarbonate ion HCO_3^- while decreasing that of carbonate ion CO_3^{2-}. These changes in the distribution of species of dissolved inorganic carbon would result in an increase in proton concentration to decrease the pH.[10)] In the carbonate buffer system, $CaCO_3$ would play an important role in keeping a constant pH by the reaction of the minerals with the excess acid. Meanwhile, the detailed effects of CO_2 pressurization on the pH control and efficiency of enzymatic saccharification in the CaCCO process are not clear, due to heterogeneity of the reaction system and pH-dependent enzyme activities during saccharification. Herein, we investigated these effects using $Ca(OH)_2$-pretreated rice straw as a substrate and a mixture of fungal enzymes for saccharification.

Fine powder of sun-dried rice straw (*cv.* Koshihikari),[11)] whose cellulose and xylan contents of the absolutely dried rice straw powder were 35.0 and 14.1 %, respectively, was used as a feedstock. The experiment for investigating the effect of CO_2 pressurization on the pH of slurry with $Ca(OH)_2$-pretreated/CO_2-neutralized (CaCCO-treated) rice straw powder was performed in a 2 L pressure-resistant reactor with a helical impeller (a custom-made reactor; Taiatsu Techno Corp., Tokyo, Japan). As shown in Fig. 1, the pH of slurry decreased with increasing pCO_2. For

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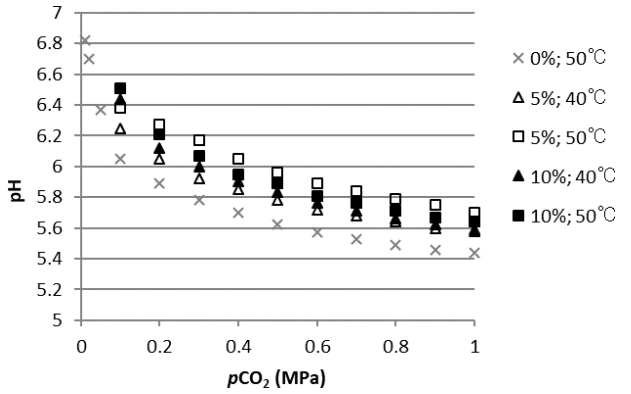

Fig. 1. Effect of CO_2 partial pressure (pCO_2) on pH of slurry with the CaCCO-treated rice straw powder.

A mixture of rice straw powder [*cv.* Koshihikari, 0.5-mm-mesh pass; 0 g for 0 % (w/w), 90 g for 5 % (w/w), 180 g for 10 % (w/w)], $Ca(OH)_2$ (18 g), and distilled water [1.8 L for 0 % (w/w), 1.71 L for 5 % (w/w), 1.62 L for 10 % (w/w)] was heated at 120 °C for 90 min. After cooling, the slurry was poured into a 2 L pressure-resistant reactor with a helical impeller. The reactor was then immersed in a heated water bath to keep the temperature of the slurry at an appropriate level (40 or 50 °C), and the CO_2 was injected into the reactor for neutralization of the slurry until its pH was equilibrated. The pH of the slurry was monitored using the sensor InPro4800SG/225/PT1000 (Mettler Toledo, Tokyo, Japan), which was installed with the reactor. After neutralization, the reactor was pressurized up to the desired partial pressure of CO_2 and the pH was measured after equilibrium was achieved.

example, pH values under the 0.1 and 1.0 MPa pressures of pCO_2 were 6.38 and 5.70, respectively, in the case of the 5 % (w/w) slurry at 50 °C. Figure 1 also indicates that the pH values are affected by the temperature. The pH values at 40 °C are lower than those at 50 °C at the same pCO_2; this can be explained by the higher solubility of $CaCO_3$ at lower temperature. A similar trend was obtained in the 10 % (w/w) slurry, where pH values at 50 °C under the 0.1 and 1.0 MPa pressures of pCO_2 were 6.51 and 5.64, respectively. All pH values of the slurry of pretreated rice straw powder were higher than those without biomass at the same pCO_2 ["0 % 50 °C" in Fig. 1; the process was carried out using 10 %(w/w) $Ca(OH)_2$ suspension]. Under the atmospheric conditions of $pCO_2 = 4 \times 10^{-5}$ MPa, the pH of the slurry ranged between 8.3 and 8.6 (data not shown). Although we have not confirmed the pH at pCO_2 lower than 0.1 MPa yet, we expect that the slurry pH of the CaCCO-treated feedstock could be controlled between 5.5 and 8.5 by changing the pCO_2 from atmospheric levels to 1.0 MPa. In comparison to the case with concentrated acid or base solution added to the slurry to control the pH, CO_2 pressurization with mechanical power would enable us to easily and reversibly control pH in both directions within the working range.

Next, we carried out an enzymatic hydrolysis experiment of $Ca(OH)_2$-pretreated/water-washed rice straw powder to determine the pH range for effective saccharification. The substrate powder was prepared by HCl neutralization of the $Ca(OH)_2$-pretreated rice straw powder and washing of the solid part with water.[7] Cellulose and xylan contents of this pretreated/washed rice straw powder were 35.8 and 15.6 %, respectively, on a dry basis. A mixture of two fungal enzyme preparations, namely, a cellulase preparation with high activity of hemicellulases from *Trichoderma reesei* M2-1[12] [12 filter-paper-degrading units (FPUs)/g-dry weight biomass at pH 5.0] and a commercial β-glucosidase preparation from *Aspergillus niger* [Novozyme 188, Novozymes Japan (Chiba, Japan); 43 cellobiase units (CbUs)/g-dry weight biomass at pH 5.0], was used as hydrolytic enzymes. The former preparation was obtained by the cultivation of M2-1 with continuous feeding of a mixed solution of glucose, xylose, and cellobiose described by Ike *et al.*[13] Figure 2 shows the sugar yields after enzymatic saccharification under various pH conditions. More soluble sugars from glucan and xylan were yielded at conditions of pH 5.0–6.0 and pH around 5.5–6.0, respectively. At pH above 6.0, the sugar yields decreased and the liberated monosaccharides in particular were at a significantly low level at pH 7.0. These results indicate that pH of 5.5–6.0 is suitable for the saccharification of $Ca(OH)_2$-pretreated/water-washed rice straw powder.

Next, we examined the enzymatic saccharification of CaCCO-treated rice straw powder under the CO_2-pressurized conditions to investigate the effect of pCO_2 on the sugar yield after enzymatic saccharification. As shown in Fig. 3, the saccharification ratio increased as the pCO_2 increased. The maximum amount of total liberated sugars from glucan and xylan (mono- and oligosaccharides) was achieved at the pCO_2 of 0.4 MPa and over, while that of monosaccharides (glucose and xylose) was obtained at the pCO_2 of 0.5 MPa. Enzymatic saccharification in this experiment was performed at the slurry concentration of 5 % (w/w) and 50 °C; the pH of the slurry at the pCO_2 of 0.4–0.5 MPa under these saccharification conditions is expected to be 6.0, according to the data in Fig. 1. This value is consistent with the result of Fig. 2 that pH of 5.5–6.0 is suitable for enzymatic saccharification.

In this study, a mixture of enzymes from two fungi was adopted for saccharification; the optimal pH for its cellulolytic (filter-paper-degrading and endoglucanase) and xylanolytic (birchwood-xylan-degrading) activities was 5.0 (data not shown). Meanwhile, *T. reesei* possesses at least six genes for xylanases, and two xylanases, XYN II and XYN III, which are major xylanases in the cellulase preparation, possess the optimum pH of 5.5–6.0.[14)15)16)17)18)] It has also been reported that xylan removal strongly affects the efficiency of cellulose hydrolysis, and there is synergy between some types of cellulases and xylanases for the degradation of lignocellulosic biomass.[19)20)21)22)23)] It is speculated that some enzymes such as XYN II and XYN III might contribute more significantly to hydrolysis at pH 5.5–6.0 than at other pH levels, which could increase the accessibility of cellulases to the substrate, resulting in the maximum sugar yield.

In summary, we elucidated the effect of CO_2 pressurization on pH control and enzymatic saccharification of $Ca(OH)_2$-pretreated rice straw powders, and determined the minimum pressure for saccharification with fungal enzymes. Taking into account that this CO_2-pressurizing system could control pH of the slurry between 5.5 and 8.5, potential applications of this system would include the use

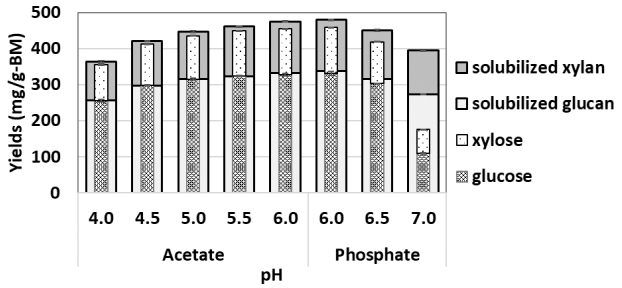

Fig. 2. Sugar yields after 24 h of enzymatic saccharification of $Ca(OH)_2$-pretreated/water-washed rice straw powder under various pH conditions.

The $Ca(OH)_2$-pretreated/water-washed pretreated rice straw (50 mg on a dry basis) was added to 1 mL of enzyme solution in the 50 mM buffer with different pH (acetate buffer pH 4.0, 4.5, 5.0, 5.5, and 6.0; phosphate buffer pH 6.0, 6.5, and 7.0). A mixture of the cellulase preparation from *T. reesei* M2-1 (0.6 FPU at pH 5.0) and Novozyme188 (2.2 CbU at pH 5.0) was used as saccharification enzyme. All saccharification reactions were performed at 50 °C for 24 h. The amounts of liberated monosaccharides (glucose and xylose) and solubilized sugars (glucose + glucose-containing oligosaccharides and xylose + xylose-containing oligosaccharides) during saccharification were measured by HPLC, as described in our previous report.[13)]

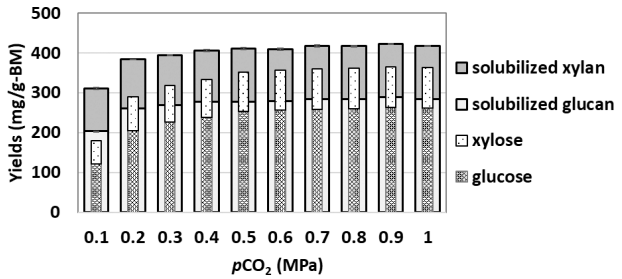

Fig. 3. Sugar yields after 24 h of CO_2-pressurized enzymatic saccharification of CaCCO-treated rice straw powder.

All reactions including $Ca(OH)_2$ pretreatment, CO_2 neutralization, and enzymatic saccharification were carried out in the 96 mL pressure-resistant glass tube "Hiper Glass Cylinder (HGC)" (HPG-96-3; Taiatsu Techno Corp.). One gram of fine-powdered rice straw, 100 mg of $Ca(OH)_2$, and 18 mL of water in the HGC were well mixed and heated at 120 °C for 1 h. After cooling, CO_2 was injected into the HGC at a pressure of 0.5 MPa and settled at room temperature overnight to complete neutralization of the slurry. Then, 1 mL of the same enzyme mixture in Fig. 2 was added to the neutralized slurry in the HGC, followed by pressurization of CO_2 up to the desired partial pressure. Saccharification reactions were performed at 50 °C for 24 h and the amounts of liberated monosaccharides and solubilized sugars were measured in the same way as for Fig. 2.

of other enzymes with pH optima within the controllable range, the use of two-step conversion of substrate with two kinds of enzymes with distinct pH optima (like α-amylase and glucoamylase for starch saccharification), simple pH-controlled bioconversion and/or slurry circulation without oxygen, the use of CO_2 in solution as substrate for its fixation, and dynamic pH shifts for changing the solubility of contaminants or products like lignin and polyphenols. While the effect of solubilization of calcium ions in the presence of large amounts of CO_2 in solution was not obvious in this study, the potential impact of ionic strength and/or direct effects of calcium ions on bio-catalytic activities should be considered for maximizing the yields.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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REFERENCES

1) R.A. Sheldon: Green and sustainable manufacture of chemicals from biomass: state of the art. *Green Chem.*, **16**, 950–963 (2014).
2) Q.G. Zhang, J.J. Hu, and D.J. Lee: Pretreatment of biomass using ionic liquids: research updates. *Renewable Energy*, **111**, 77–84 (2017).
3) A.K. Chandel, V.K. Garlapati, A.K. Singh, F.A.F. Antunes, and S.S. da Silva: The path forward for lignocellulose biorefineries: Bottlenecks, solutions, and perspective on commercialization. *Bioresour. Technol.*, **264**, 370–381 (2018).
4) S.S. Hassan, G.A. Williams, and A.K. Jaiswal: Emerging technologies for the pretreatment of lignocellulosic biomass. *Bioresour. Technol.*, **262**, 310–318 (2018).
5) F.M. Gírio, C. Fonseca, F. Carvalheiro, L.C. Duarte, S. Marques, and R. Bogel-Łukasik: Hemicelluloses for fuel ethanol: A review. *Bioresour. Technol.*, **101**, 4775–4800 (2010).
6) P. Alvira, E. Tomás-Pejó, M. Ballesteros, and M.J. Negro: Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour. Technol.*, **101**, 4851–4861 (2010).
7) J.Y. Park, R. Shiroma, M.I. Al-Haq, Y. Zhang, M. Ike, Y. Arai-Sanoh, A. Ida, M. Kondo, and K. Tokuyasu: A novel lime pretreatment for subsequent bioethanol production from rice straw – Calcium capturing by carbonation (CaCCO) process. *Bioresour. Technol.*, **101**, 6805–6811 (2010).
8) R. Shiroma, J.Y. Park, M.I. Al-Haq, M. Arakane, M. Ike, and K. Tokuyasu: RT-CaCCO process: An improved CaCCO process for rice straw by its incorporation with a step of lime pretreatment at room temperature. *Bioresour. Technol.*, **102**, 2943–2949 (2011).
9) M. Ike, R. Zhao, M.S. Yun, R. Shiroma, S. Ito, Y. Zhang, Y. Zhang, M. Arakane, M.I. Al-Haq, J. Matsuki, J.Y. Park, M. Gau, K. Yakushido, M. Nagashima, and K. Tokuyasu: High solid-loading pretreatment/saccharification tests with CaCCO (calcium capturing by carbonation) process for rice straw and domestic energy crop, *Erianthus arundinaceus*. *J. Appl. Glycosci.*, **60**, 177–185 (2013).
10) D. Shi, Y. Xu, and F.M.M. Morel: Effects of the pH/pCO_2 control method on medium chemistry and phytoplankton growth. *Biogeosciences*, **6**, 1199–1207 (2009).

11) R. Zhao, M.S. Yun, R. Shiroma, M. Ike, D. Guan, and K. Tokuyasu: Integration of a phenolic-acid recovery step in the CaCCO process for efficient fermentable-sugar recovery from rice straw. *Bioresour. Technol.*, **148**, 422–427 (2013).
12) M. Ike, J.Y. Park, M. Tabuse, and K. Tokuyasu: Cellulase production on glucose-based media by the UV-irradiated mutants of *Trichoderma reesei*. *Appl. Microbiol. Biotechnol.*, **87**, 2059–2066 (2010).
13) M. Ike, J.Y. Park, M. Tabuse, and K. Tokuyasu: Controlled preparation of cellulases with xylanolytic enzymes from *Trichoderma reesei* (*Hypocrea jecorina*) by continuous-feed cultivation using soluble sugars. *Biosci. Biotechnol. Biochem.*, **77**, 161–166 (2013).
14) M. Tenkanen, J. Puls, and K. Poutanen: Two major xylanases of *Trichoderma reesei*. *Enzyme Microb. Technol.*, **14**, 566–574 (1992).
15) J. Xu, N. Takakuwa, M. Nogawa, H. Okada, and Y. Morikawa: A third xylanase from *Trichoderma reesei* PC-3-7. *Appl. Microbiol. Biotechnol.*, **49**, 718–724 (1998).
16) M. Tenkanen, M. Vršanská, M. Siika-aho, D.W. Wong, V. Puchart, M. Penttilä, M. Saloheimo, and P. Biely: Xylanase XYN IV from *Trichoderma reesei* showing exo- and endo-xylanase activity. *FEBS J.*, **280**, 285–301 (2013).
17) J. Ramoni, M. Marchetti-Deschmann, V. Seidl-Seiboth, and B. Seiboth: *Trichoderma reesei* xylanase 5 is defective in the reference strain QM6a but functional alleles are present in other wild-type strains. *Appl. Microbiol. Biotechnol.*, **101**, 4139–4149 (2017).
18) P. Biely, V. Puchart, M.A. Stringer, and K.B. Mørkeberg Krogh: *Trichoderma reesei* XYN VI - a novel appendage-dependent eukaryotic glucuronoxylan hydrolase. *FEBS J.*, **281**, 3894–3903 (2014).
19) B. Yang and C.E. Wyman: Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. *Biotechnol. Bioeng.*, **86**, 88–95 (2004).
20) R. Kumar and C.E. Wyman: Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. *Biotechnol. Prog.*, **25**, 302–314 (2009).
21) M.J. Selig, W.S. Adney, M.E. Himmel, and S.R. Decker: The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes. *Cellulose*, **16**, 711–722 (2009).
22) J. Zhang and L Viikari: Impact of xylan on synergistic effects of xylanases and cellulases in enzymatic hydrolysis of lignocelluloses. *Appl. Biochem. Biotechnol.*, **174**, 1393–1402 (2014).
23) S. Oladi and G.M. Aita: Interactive effect of enzymes and surfactant on the cellulose digestibility of un-washed and washed dilute ammonia pretreated energy cane bagasse. *Biomass and Bioenergy*, **109**, 221–230 (2018).