Evaluation of Two Sets of Sorghum Bagasse Samples as the Feedstock for Fermentable Sugar Recovery via the Calcium Capturing by Carbonation (CaCCO) Process

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Abstract: Sorghum bagasse samples from two sets (n6 and bmr6; n18 and bmr18) of wild-type and corresponding “brown midrib” (bmr) mutant strains of sweet sorghum were evaluated as the feedstock for fermentable sugar recovery via the calcium capturing by carbonation (CaCCO) process, which involves Ca(OH)2 pretreatment of bagasse with subsequent neutralization with CO2 for enzymatic saccharification. Saccharification tests under various pretreatment conditions of the CaCCO process at different Ca(OH)2 concentrations, temperatures or residence periods indicated that bmr strains are more sensitive to the pretreatment than their counterparts are. It is expected that variant bmr6 is more suitable for glucose recovery than its wild-type counterpart because of the higher glucan content and better glucose recovery with less severe pretreatment. Meanwhile, bmr18 showed higher scores of glucose recovery than its counterpart did, only at low pretreatment severity, and did not yield higher sugar recovery under the more severe conditions. The trend was similar to that of xylose recovery data from the two bmr strains. The advantages of bmr strains were also proven by means of simultaneous saccharification and fermentation of CaCCO-pretreated bagasse samples by pentose-fermenting yeast strain Candida shehatae Cs 4R. The amounts needed for production of 1 L of ethanol from n6, bmr6, n18, and bmr18 samples were estimated as 4.11, 3.46, 4.03, and 3.95 kg, respectively. The bmr strains seem to have excellent compatibility with the CaCCO process for ethanol production, and it is expected that integrated research from the feedstock to bioprocess may result in breakthroughs for commercialization.

Key words: bioethanol, CaCCO, RT-CaCCO, sorghum, brown midrib

INTRODUCTION

Bioethanol—fuel ethanol made from biomass—is regarded as a promising renewable energy resource and a possible alternative to fossil fuels.1 In particular, the production of bioethanol from lignocellulosic biomass is considered a key technology preventing the competition with food production.2 Lignocellulosic biomass generally requires pretreatment to alter its structure and to make cellulose and hemicellulose—the major polysaccharides—more accessible to the enzymes that convert them into fermentable sugars.3 Several studies have been published on the pretreatment of lignocellulosic biomass; they can be subdivided into physical, chemical, physicochemical, and biological processes.4–9

Sorghum [Sorghum bicolor (L.) Moench] has attracted interest as a resource for bioethanol production because of its rapid growth and its high biomass recovery from the field.8–9 Sorghum mainly contains glucose, fructose, sucrose, and starch as nonstructural carbohydrates.8 These carbohydrates can also serve as sources for bioethanol production.9,10 Brown midrib (bmr) mutants were developed in sorghum,10 and these phenotypes are generally described as reduced lignin content, altered lignin chemical composition and greater sensitivity to lodging as compared with their wild-type counterparts.11 These phenotypes are known to be derived from the reduced activity of lignin biosynthetic enzymes such as cinnamyl alcohol dehydrogenases (CADs) or caffeic O-methyl transferases (COMTs).12 Feeding of
bmr mutants of sorghum to dairy cows has been reported to have advantages for milk production, ruminal fiber digestibility and enzymatic digestibility over their wild-type counterparts without altered lignin biosynthesis.\textsuperscript{21}\textsuperscript{23} Wu et al.\textsuperscript{19} observed strong capacity of a bmr mutant, Kyushuuko 4, for enzymatic degradability after pretreatment of the sorghum bagasse with sodium hydroxide. Maehara \textit{et al.}\textsuperscript{20} reported that the bmr mutant has superior enzymatic digestibility of fibers after pretreatment with calcium hydroxide in comparison with the wild-type counterpart. However, these processes include washing steps for removal of the alkali reagents used for pretreatments; these steps result in the loss of xylan. Xylose is the main hydrolysis product of xylan, and this fermentable pentose sugar is regarded as an alternative to glucose for bioethanol production and for efficient utilization of the feedstock.\textsuperscript{21}

Recently, we developed a novel lime (\textit{Ca(OH)}\textsubscript{2})-pretreatment process: CaCCO or “calcium capturing by carbonation (CO\textsubscript{2})”.\textsuperscript{11} Its standard pretreatment conditions, 120 °C for 1 h, were modified to make them less harsh: ambient temperature for >7 days, and this advanced process was termed RT-CaCCO (CaCCO at room temperature).\textsuperscript{19} In these processes, the reaction mixture after the \textit{Ca(OH)}\textsubscript{2} pretreatment is directly neutralized by CO\textsubscript{2}, gas, and the generated CaCO\textsubscript{3} is entrapped in the reaction vessel during the saccharification/fermentation processes. The use of CO\subscript{2} for neutralization may reduce the cost of acid reagents, because CO\textsubscript{2} could be available as a byproduct from the fermentation/combustion processes. The solid parts of the distillery could be burned to generate both heat energy and inorganic salts for CO\textsubscript{2} recovery. The process could obviate the solid-liquid-separation and washing steps, resulting in a one-pot system for bioethanol production. It is notable that this one-pot process could enable researchers to keep solubilized sugars, such as xylan, sucrose and starch, in the vessel. In addition, the \textit{Ca(OH)}\textsubscript{2} pretreatment in the RT-CaCCO process can be regarded as a wet preservation step of the biomass at ambient temperature and high pH.

Here, we evaluated the saccharification efficiency for sorghum bagasse subjected to the CaCCO or RT-CaCCO process and the ethanol production by a yeast strain, \textit{Candida shehatae}, which converts both glucose and xylose to ethanol. Sorghum cultivars \textit{bmr6} and \textit{bmr18} as well as their wild-type counterparts (\textit{n6} and \textit{n18}, respectively) were used in this study. We also reviewed severity of the pretreatment for these samples and differences between wild-type cultivars and their \textit{bmr} mutants as the feedstock for bioethanol.

**MATERIALS AND METHODS**

**Materials.** Sorghum (strains \textit{n6}, \textit{bmr6}, \textit{n18}, and \textit{bmr18}) was harvested in October 2010 in an experimental field of the National Agricultural Research Center, NARO, Tsukuba, Japan. The \textit{bmr6} strain has a mutation principally affecting CAD activity.\textsuperscript{22} In contrast, \textit{bmr18} has a mutation in the COMT gene.\textsuperscript{21} The heads and leaves were removed from the sorghum samples. The stalks of these samples were chopped into 50-cm pieces, and then the cut stalks were squeezed using a sugarcane milling machine [TM-340 (B), Matsuo Co., Ltd., Kagoshima, Japan]. Sorghum bagasse, the residue after squeezing of the sorghum stalks, was washed with distilled water until free sugars were no longer detected by the Glucose C-II Test Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in the washing liquor, and the washed bagasse was dried at 70 °C for 3 days in a drying oven. The dried sorghum bagasse was milled using a multimill machine (RDI-15, Grow Engineering Co., Ltd., Tokyo, Japan) equipped with hammer mill blades so that the bagasse could pass through a 500-μm mesh sieve and could be used as a sorghum bagasse powder. Other chemicals were reagent grade commercial products.

**Analytical methods.** The amounts of structural compounds in the cell walls of the sorghum bagasse powders were measured as described elsewhere.\textsuperscript{19} A sorghum bagasse powder (200 mg) was mixed with 2 mL of 72 % (w/w) H\textsubscript{2}SO\textsubscript{4}, and the mixture was stirred well using a glass rod. After incubation of the mixture in a water bath at 30 °C for 1 h, distilled water (14 mL) was added. The diluted mixture was heated at 100 °C for 2 h for dilute acid hydrolysis of sugars.

An aliquot (1.0 mL) of the acid hydrolysate was taken for analyses of total glucose, xylose and acid-soluble lignin contents of each sorghum bagasse powder. After centrifugation (20,000 × \textit{G}, 3 min, 4 °C) of the sampled hydrolysate, the supernatant (0.5 mL) was collected and neutralized with 10 % (w/v) NaOH. Total glucose and xylose contents of the supernatant were determined by means of the Glucose C-II Test Kit and D-xylose Assay Kit (Megazyme International Ireland Co., Ltd., Wicklow, Ireland, respectively). Glucan and xylan contents were calculated from the glucose and xylose contents, under the assumption that glucan and xylan are linear polymers of β-1,4-linked glucose and xylose residues, respectively. The correction coefficients for hydration of cellulose and xylan were 0.90 and 0.88, respectively. The amount of acid-soluble lignin was determined by measuring absorbance at 205 nm in an aliquot and using the value of 110 L/(g·cm) as the absorbptivity of soluble lignin.\textsuperscript{22}

For quantitation of acid-insoluble compounds in a sorghum bagasse powder, the mixture after the dilute acid hydrolysis was centrifuged (8,000 × \textit{G}, 10 min, 4 °C), and the supernatant was removed. Then, the precipitate was washed with 5 mL of distilled water by thorough mixing on a vortex mixer. The mixed sample was centrifuged (8,000 × \textit{G}, 10 min, 4 °C), and the supernatant was removed. This step (washing of the precipitate) was repeated five times. The washed precipitate was dried at 105 °C to constant weight, and then combusted at 600 °C for 5 h in a muffle furnace. After cooling down to room temperature, the remaining ash was weighed as the acid-insoluble ash. The amount of acid-insoluble lignin was calculated by subtracting the weight of acid-insoluble ash from that of the dried precipitate. A sorghum bagasse powder (100 mg) was combusted at 600 °C for 5 h in a muffle furnace. After cooling down to room temperature, the remaining ash was weighed as the total ash content.

\textit{Ca(OH)}\textsubscript{2} pretreatment/neutralization of sorghum bagasse via a CaCCO or RT-CaCCO process. The optimization
of Ca(OH)₂ pretreatment for the CaCCO process was performed using any of the following three processes. (i) A sorghum bagasse powder (200 mg) was placed in a 10-mL glass vial. Ca(OH)₂, ranging from 0 to 30 mg [0–15 % (w/w to sorghum bagasse powder)] and distilled water (4 mL) were added to the vial, and the contents were mixed well on a vortex mixer. After sealing the vial with a butyl-rubber-and-aluminum cap, we heated the mixture in the vial at 120 °C for 1 h in an oil bath and cooled it on the bench to room temperature. (ii) A sorghum bagasse powder (200 mg) was placed in a 10-mL glass vial. Ca(OH)₂ (20 mg) and distilled water (4 mL) were added to the vial, and the contents were mixed well on a vortex mixer. After sealing the vial with a butyl-rubber-and-aluminum cap, we heated the mixture in the vial at 120 °C from 5 to 90 min in an oil bath and cooled it on the bench to room temperature. (iii) A sorghum bagasse powder (200 mg) was placed in a 10-mL glass vial. Ca(OH)₂ (20 mg) and distilled water (4 mL) were added to the vial, and the contents were mixed well on a vortex mixer. After sealing the vial with a butyl-rubber-and-aluminum cap, we heated the mixture in the vial at 120 °C from 5 to 90 min in an oil bath and cooled it on the bench to room temperature.

For the RT-CaCCO process, a sorghum bagasse powder (200 mg) was placed in a 10-mL glass vial, and distilled water (4 mL) was added to the vial with or without Ca(OH)₂ (20 mg). After sealing the vial with a butyl-rubber-and-aluminum cap, we mixed the mixture in the vial well on a vortex mixer. The vial was stored at room temperature for 7 days.

After each pretreatment for the CaCCO or the RT-CaCCO process, the headspace of the vial was aseptically exchanged with filtered CO₂ purified through inlet and outlet needles. After the outlet needle was removed, inner CO₂ pressure of the vial was maintained at 0.15 MPa through the inlet needle for 20 min. The neutralized sample was used for evaluation of monomeric-sugar recovery after enzymatic saccharification.²⁵

Enzymatic saccharification. Activities of the main enzymes in the enzyme preparations were evaluated beforehand in 50 mM phosphate buffer, pH 6.5, at 50 °C, so as to predict their actual activities at pH in the presence of CaCO₃.²⁹ A solution of a mixture of enzymes (1 mL) was added to the Ca₂⁺-neutralized sample in the vial. The filter paper-degrading (FPU) and cellulose activities (CbU) were measured according to the IUPAC recommendations.²³ Xylanase activity toward birchwood xylan (Sigma Chemical Co., St. Louis, USA) was determined by quantifying reducing sugars produced during the enzymatic reaction using the Somogyi-Nelson method.²⁴ Two units of xylanase activity was defined as the amount of the enzyme that produced 1 μmol of reducing sugars per minute in xylose equivalents.

The activities of individual enzymes were as follows: Cellulast 1.5 L (Novozymes Japan, Chiba, Japan); 12 FPU per gram of an untreated sorghum bagasse powder at pH 6.5, Novozyme 188 (Novozymes Japan); 7.2 CbU per gram of an untreated sorghum bagasse powder at pH 6.5, Ultraflo L (Novozymes Japan); 40 U xylanase and 20 CbU per gram of an untreated sorghum bagasse powder at pH 6.5.

The concentrations of glucose, xylose and ethanol were measured by high-performance liquid chromatography using a Shimadzu LC-20 system (Shimadzu Co., Ltd., Tokyo, Japan) with an RI detector (RID-10A, Shimadzu). An Aminex HPX-87 ion exclusion column (Bio-Rad Laboratories, Inc., Hercules, USA) was used at 50 °C. The elution buffer was 5 mM sulfuric acid, and the flow rate for the measurement was 0.6 mL/min.

Microorganisms. A mutant named Cs 4R was used for fermentation; it was derived by mutagenesis from Candida shehatae ATCC 22984 (obtained from the American Type Culture Collection) and screened by 2,3,5-triphenyltetrazolium chloride and 2-deoxy-D-glucose. Previously, the mutant Cs 3512 was derived from C. shehatae ATCC 22984, which produces 8 % more ethanol than C. shehatae ATCC 22984 does.²⁰ Cs 4R was further mutated (derived from Cs 3512) and showed faster ethanol production than Cs 3512 did (data not shown). The Cs 4R cells were stored on YPM-X agar plates at 4 °C, which were composed of YPM [per liter: Bacto peptone (Difco Laboratories, Detroit, USA) 5 g, Bacto yeast extract (Difco Laboratories) 3 g, Bacto malt extract (Difco Laboratories) 3 g, CaCl₂ 200 mg, KH₂PO₄ 2.5 g, MgSO₄·7H₂O 500 mg, (NH₄)₂SO₄ 1 g, 10 g/L xylose, and 20 g/L Bacto agar (Difco laboratories)]. For a precul- ture, Cs 4R was grown in the YPM-X (with 10 g/L xylose) medium at 30 °C and 250 rpm for 24 h. The cells of Cs 4R were pelleted by centrifugation (3,000 × G, 5 min), washed twice with 0.8 % (w/v) NaCl saline, and resuspended in the appropriate amount of distilled water prior to inoculation.

The concentration of microorganisms was measured by optical density at 600 nm (OD₆₀₀) in a 1-cm cuvette on a spectrometer (SpectraMax Plus 384, Molecular Devices, Tokyo, Japan). OD₆₀₀ of Cs 4R cells was used to estimate the concentration: 0.22 g/L dry cells (our unpublished data).

Simultaneous saccharification and fermentation (SSF) of sorghum bagasse via CaCCO process. A sorghum bagasse powder (0.5 g, at solid concentration 5 % w/w) was treated by the CaCCO process [10 % (w/w dry sorghum bagasse powder) Ca(OH)₂, 120 °C, 1 h], and then the SSF was performed in a glass vial (50 mL) capped with a butyl rubber stopper. The glass vial was connected to the atmosphere through a 0.2-µm-pore filter (Millipore, Millipore, Carrigtwohill Co., Cork, Ireland), which was fixed on a syringe needle (18 G × 1 ½", Terumo Co., Ltd., Tokyo, Japan) inserted into the rubber stopper. The SSF was started by addition of enzyme mixtures (in the same amounts as described above) and 224 µL of the Cs 4R inoculum (corresponding to 49.3 µg dry cells). The glass vial was shaken at 30 °C and 250 rpm in a rotary shaker (BR-23FP, TAITEC, Tokyo, Japan). Aliquots of the samples (0.1 mL) were taken by the syringe at various time points for analysis of the concentration of glucose, xylose and ethanol.

The concentrations of glucose, xylose and ethanol were measured using a Shimadzu LC-20 system (Shimadzu Co., Ltd., Tokyo, Japan) with an RI detector (RID-10A, Shimadzu Co., Ltd.)

²⁴ An Aminex HPX-87 ion exclusion column (Bio-Rad Laboratories, Inc., Hercules, USA) was used at 50 °C. The elution buffer was 5 mM sulfuric acid, and the flow rate for the measurement was 0.6 mL/min.
RESULTS AND DISCUSSION

Main components of bagasse samples.

Table 1 shows the main components in two sets of dry bagasse powder samples. As for the set of n6 and bmr6, significant differences in both glucan contents and lignin contents were observed, which were not so obvious between n18 and bmr18. The higher glucan content and lower lignin content of bmr6 relative to n6 were in agreement with the results from reports by Maehara et al.18 and Lam et al.22, respectively.

The sorghum bagasse samples of the wild type and bmr mutants, used in this study, were prepared under the same cultivation conditions, with the only difference being the bmr mutation in the genome. Therefore, the differences in glucan and lignin contents can be attributed to the bmr mutation of sorghum, which may improve the feedstock quality for recovery of fermentable sugars.

Effects of Ca(OH)2 concentration, temperature and residence time during pretreatment on enzymatic saccharification via the CaCCO process.

For a set of n6 and bmr6 bagasse powders, the recovery levels of glucose and xylose after enzymatic saccharification following pretreatment at different Ca(OH)2 concentrations are shown in Figs. 1A and B, respectively. In both samples, sugar recovery levels were mostly constant at the Ca(OH)2 concentrations above 7.5 % (w/w dry bagasse powder). The recovery levels of glucose and xylose from bmr18 tended to be slightly higher than those from n18 when the Ca(OH)2 concentrations were below 7.5 % (w/w dry bagasse powder). The differences in the maximal sugar recovery between n18 and bmr18 were not easily detectable at the higher concentrations, suggesting that the feedstock characteristics were similar in terms of both the components (Table 1) and pretreatment efficiency when high recovery of fermentable sugars is needed.

The effect of temperature during pretreatment on the sugar yield after enzymatic saccharification was also analyzed (Fig. 2). As shown in Figs. 2A and B, the recovery of glucose and xylose from n6 reached the maximum at >100 °C. Meanwhile, the corresponding values from bmr6 reached the maximum at a much lower temperature (60 °C).

The pretreatment of bmr6 at room temperature (25 °C) for 1 h yielded high recovery of glucose and xylose: 70.0 and 65.0 %, respectively. Figs. 2C and D shows the cases of n18 and bmr18, and the recovery levels of glucose and xylose increased at elevated temperatures and appeared to reach the maximum at >100 °C. The recovery of glucose and xylose from bmr18 was higher in comparison with n18 at all the temperatures tested. The bmr6 showed higher sensitivity to alkaline treatment than bmr18 did, and the significant effect of pretreatment of bmr6 was observed even with the Ca(OH)2 pretreatment at room temperature (25 °C) for 1 h. It is expected that the phenotype of bmr6 will help to reduce heating energy during pretreatment. It is noteworthy that the increases in glucose recovery in all samples slowed down and reached a plateau at ~100 °C (Figs. 2A and C), whereas increases in xylose recovery reached a plateau at a higher temperature: ~120 °C (Figs. 2B and D). The difference in the trends suggests that high recovery of xylose requires more severe conditions, and the increase in xylose recovery will not always result in the increase of glucose recovery.

Next, we evaluated the effects of residence time during the pretreatment of n6 and bmr6 at 120 °C and at Ca(OH)2 concentration of 10 % (w/w of dry bagasse powder) (Figs. 3A and B). We found that the recovery levels of glucose and xylose from bmr6 were higher than those from n6 for all residence periods. The recovery of glucose and xylose...
Sugar recovery after enzymatic saccharification of CaCCO-pretreated sorghum bagasse powders at various Ca(OH)$_2$ concentrations (120 °C, 1 h). The error bars represent standard deviation of triplicate values.

A: Glucose recovery from strains $n_6$ and $bmr_6$; B: xylose recovery from $n_6$ and $bmr_6$; C: glucose recovery from $n_18$ and $bmr_{18}$; D: xylose recovery from $n_18$ and $bmr_{18}$.

Fig. 1. Sugar recovery after enzymatic saccharification of CaCCO-pretreated sorghum bagasse powders at various temperatures (10 % Ca(OH)$_2$; w/w dry bagasse powder, 1 h). The error bars represent standard deviation of triplicate values. A: Glucose recovery from strains $n_6$ and $bmr_6$; B: xylose recovery from $n_6$ and $bmr_6$; C: glucose recovery from $n_18$ and $bmr_{18}$; D: xylose recovery from $n_18$ and $bmr_{18}$.

Fig. 2. Sugar recovery levels after enzymatic saccharification of a CaCCO-pretreated sorghum bagasse powder at various temperatures (10 % Ca(OH)$_2$; w/w dry bagasse powder, 1 h). The error bars represent standard deviation of triplicate values. A: Glucose recovery from strains $n_6$ and $bmr_6$; B: xylose recovery from $n_6$ and $bmr_6$; C: glucose recovery from $n_18$ and $bmr_{18}$; D: xylose recovery from $n_18$ and $bmr_{18}$.
from bmr6 at the short residence time of 5 min was 75.3 and 68.8 %, respectively. As for n18 and bmr18, the recovery of glucose and xylose reached the maximum when pretreatment time exceeded 45 min. The recovery levels of glucose and xylose from bmr18 were slightly higher than those of n18 for most of the residence periods tested. The glucose recovery of the wild-type sorghum bagasse powder gradually improved with the increase in residence time. On the other hand, the glucose recovery from the bmr mutants reached a plateau at the residence time of 15–45 min. The trend of xylose recovery from the bmr counterparts was mostly the same as that of glucose recovery. The recovery levels of glucose and xylose from n6 and bmr6 tended to be higher at shorter residence time (especially 5–30 min) as compared with those from n18 and bmr18 (Figs. 3C and D).

Effects of residence time during pretreatment at room temperature on enzymatic saccharification via the RT-CaCCO process.

We indicated in our previous report that Ca(OH)2 pretreatment of rice straw at room temperature for 7 days has an equivalent effect on the subsequent saccharification in comparison with that at 120 °C for 1 h in the CaCCO process.19) In this study, the effects of Ca(OH)2 pretreatment at room temperature on the saccharification yield were evaluated using sorghum bagasse powders. Two sets of sorghum bagasse powders (n6 and bmr6; n18 and bmr18) were kept at room temperature for various periods with or without Ca(OH)2, and the recovery of glucose and xylose after enzymatic saccharification was evaluated via the RT-CaCCO process (Fig. 4). A gap in the maximum glucose recovery from n6 and bmr6 in the presence of Ca(OH)2 was clearly observed (Fig. 4A), whose trend is also shown in Figs. 1, 2, and 3. Meanwhile, the corresponding gap of xylose recovery became obscure as the residence time increased (Fig. 4B). The increase in sugar recovery from n18 and bmr18 was observed at the residence time of 1 h with Ca(OH)2, and the recovery reached a plateau at the residence time of 3 days (Figs. 4C and D). The recovery levels of glucose and xylose from bmr18 were the same as or only slightly higher than those of n18 during their plateau. The glucose recovery from n6 and bmr6 by the RT-CaCCO process tended to be higher (270 g for n6 and 341 g for bmr6 of glucose equivalent per kilogram of dry bagasse powder, Fig. 4A) than that of the CaCCO process (120 °C for 1 h, 244 g for n6 and 318 g for bmr6 glucose equivalent per kilogram of dry bagasse powder, Fig. 1A). This effect may be attributed to the milder heating conditions in the RT-CaCCO process as compared with that in the CaCCO process. Slight decreases in sugar recovery under the most severe conditions in Figs. 1A, 1B, 2A, and 2B imply that the over-degradation of sugars might have occurred and reduced the recovery values. The series of samples without Ca(OH)2 had no positive effects on the sugar recovery, and in some cases, the recovery decreased as the residence time increased. Microbial contamination might have decreased the amount of available substrates for the enzymatic saccharification or might have reduced the accessible areas for the

![Graph](https://example.com/graph.png)
enzymes.

**Simultaneous saccharification and fermentation (SSF).**

The SSF experiment on a CaCCO-pretreated sorghum bagasse powder was performed with *Candida shehatae* Cs4R, a mutant strain of pentose-fermenting yeast, *C. shehatae* ATCC 22984. The results on glucose and xylose consumption and ethanol production for *n*6 and *bmr*6 are shown in Fig. 5A. The ethanol concentration produced by *bmr*6 (12.0 g/L) was significantly higher than that of *n*6 (10.1 g/L); this result may be attributed to the higher concentration of fermentable sugars in *bmr*6 than in *n*6. This finding seems to indicate better feedstock properties of *bmr*6, as shown in Figs. 1, 2, and 3. The results from SSF for *n*18 and *bmr*18 are shown in Fig. 5B, where glucose and xylose were effectively consumed within 24 and 90 h from the beginning of SSF, respectively. The ethanol concentration reached almost the maximum within 63 h of fermentation for both *n*18 and *bmr*18. The ethanol concentra-
tion produced by \textit{bmr18} was almost equivalent to that of \textit{n18}. According to the results of Fig. 5, the weights of sorghum bagasse powders (\textit{nb}, \textit{bmr6}, \textit{n18}, and \textit{bmr18}) needed for production of 1 L of ethanol were estimated as 4.11, 3.46, 4.03, and 3.95 kg, respectively.

In both sets of bagasse powders, the \textit{bmr} mutants yielded better scores (lower weights per 1 L of ethanol production) than their wild-type counterparts; strain \textit{bmr6} showed the best properties as a feedstock for ethanol production via the CaCCO process with SSF; this finding could be due to both its high concentration of polysaccharides and its high sensitivity to Ca(OH)$_2$ pretreatment.

We examined the suitability of two sets of sorghum bagasse powders (\textit{nb} and \textit{bmr6}, \textit{n18}, and \textit{bmr18}) as a feedstock for bioethanol production and proved that \textit{bmr} mutants have better qualities than their wild-type counterparts, in terms of polysaccharide content, sensitivity to Ca(OH)$_2$ pretreatment and ethanol yields via the CaCCO process with SSF. Our laboratory tests in this study also made it clear that a single mutation of one gene that is related to lignin synthesis can dramatically improve the feedstock quality. This finding should be applied to commercially grown sorghum cultivars and other strategic herbaceous feedstocks for bioethanol production. Taking into account that the wet bagasse just after sugar juice squeezing would rapidly decay due to the microbial contamination, we propose the RT-CaCCO process for efficient preservation/pretreatment of wet bagasse.\(^{27}\) The strong applicability of \textit{bmr6} bagasse to the RT-CaCCO process should enable us to move on to further research into optimization of the bioethanol production process. Integrated research encompassing the feedstock development and efficient enzymatic saccharification is needed for detailed cost/life-cycle analyses of the whole process.

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