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Evaluation of Brown Midrib Sorghum Mutants as a Potential Biomass Feedstock for 2,3-Butanediol Biosynthesis

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Abstract Three sorghum backgrounds [Atlas, Early Hegari (EH), and Kansas Collier (KC)] and two *bmr* mutants (*bmr*6 and *bmr*12) of each line were evaluated and compared for grain and biomass yield, biomass composition, and 2,3-butanediol production from biomass. The data showed that the *bmr6* mutation in EH background led to a significant decrease in stover yield and increase in grain yield, whereas the stover yield was increased by 64% without affecting grain yield in KC background. The *bmr* mutants had 10 to 25% and 2 to 9% less lignin and structural carbohydrate contents, respectively, and 24 to 93% more non-structural sugars than their parents in all sorghum lines, except EH *bmr*12. The total fermentable sugars released were 22 to 36% more in *bmr* mutants than in parents for Atlas and KC, but not for EH. The *bmr*6 mutation in KC background produced the most promising feedstock, among the evaluated *bmr* mutants, for 2,3-butanediol production without affecting grain yield, followed by KC *bmr*12 and Atlas *bmr*6, but the *bmr* mutation had an adverse effect in EH background. This indicated that the genetic background of the parent line and type of *bmr* mutation significantly affect the biomass quality as a feedstock for biochemical production.

Keywords *Bmr* · Biomass composition · Pretreatment · Fermentation · 2,3-Butanediol · Platform chemical · Stover (dry biomass)

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Introduction

In 2013, 23.4 billion gallons of bioethanol was produced globally from maize, sugarcane, and other food materials [1]. USA alone produced 13.3 billion gallons ethanol, which consumed 30% of US maize [2]. The US Renewable Fuels Standards (RFS2) set a goal of producing 36 billion gallons of transportation fuel per year from renewable resources by 2022 [3]. In addition, a number of platform and bulk chemicals, including 2,3-butanediol, should be produced via biological routes to minimize dependency on petroleum-derived products. 2,3-Butanediol is an important platform chemical to produce a number of high-value products, including foods, pharmaceuticals, fuels, polymers, and chemicals [4–6]. Figure 1 illustrates some of the potential applications of 2,3-butanediol-derived products. The global demand for 2,3-butanediol is estimated to be around 32 million tons per year [7]. Current approaches to production of bio-based fuels and chemicals are inadequate to replace petroleum products without affecting global food supply. Therefore, abundantly available lignocellulosic biomass must be exploited for bio-based fuel and chemical production [8].

The major roadblock to the utilization of lignocellulosic biomass is the need for an energy-intensive pretreatment process prior to hydrolysis of carbohydrate polymers because of the presence of a strong outer lignin layer [9, 10]. Lignin is a heterogeneous polyphenolic polymer made up of three types of monomers, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), that are linked by carbon-carbon, ester, or ether linkages. Grass lignin also contains a considerable amount of *p*-coumarates and ferulate monomers [11]. In addition to total lignin content, composition of lignin monomers and inter-unit linkages also affect biomass

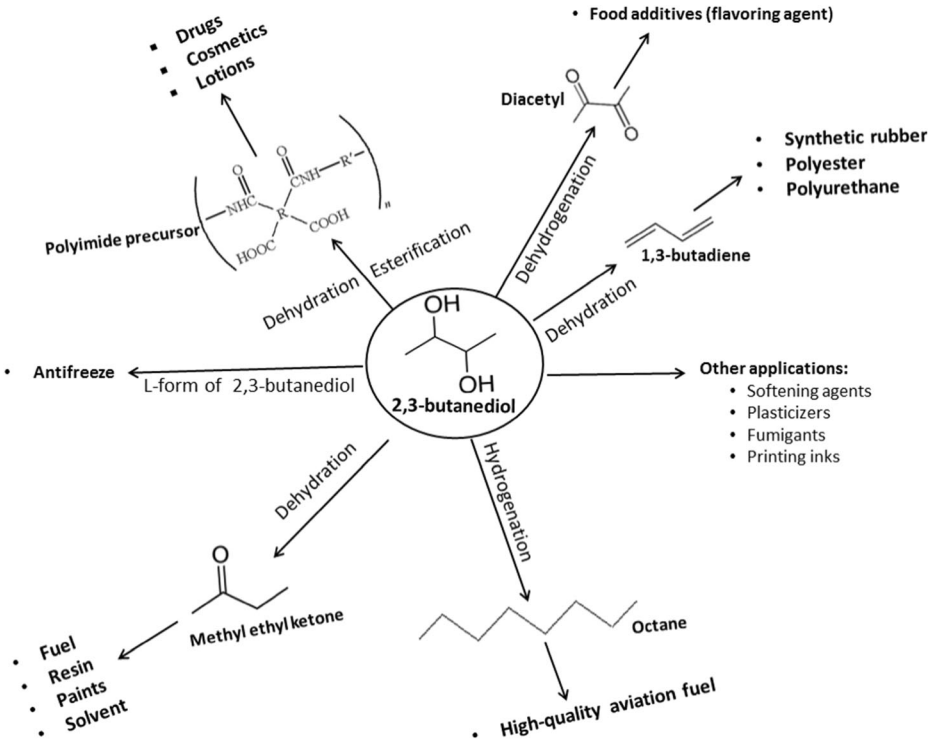

Fig. 1 Some important derivatives of 2,3-butanediol, and their potential applications [4–7]

pretreatment efficiency. Higher S/G ratio, ester, and ether linkages improve pretreatment efficiency, whereas high carbon-carbon linkages decrease it [12].

Sorghum is considered a model energy crop because of its high photosynthetic efficiency, abiotic stress tolerance, and wide applications as a food, feed, and fuel. It can be cultivated on degraded lands or infertile soils that are unfavorable for other crops, including maize [13]. The brown midrib (*bmr*) mutation of sorghum leads to decreased lignin content and altered lignin composition [13]. Phenotypically, the presence of the *bmr* gene(s) is characterized by brown coloration in mid-leaf veins in the sorghum plant [14]. Among various known *bmr* mutants, *bmr*6, *bmr*12, and *bmr*18 are agronomically acceptable in sorghum [3]. Allelic genes *bmr*12 and *bmr*18 decrease caffeic acid *O*-methyltransferase (COMT) activity, and *bmr*6 decreases cinnamyl alcohol dehydrogenase (CAD) activity [15]. As shown in Fig. 2, the COMT enzyme is responsible for a methyl group addition to 5-hydroxyconiferyl aldehyde; therefore, a decrease in the enzyme activity leads to a decrease in syringyl (S) and accumulation of the 5-hydroxy guaiacyl monomer. The CAD enzyme is responsible for a decrease in each cinnamyl aldehyde and its corresponding cinnamyl alcohol at the final step of monolignol biosynthesis. Therefore, decreased CAD enzyme activity leads to decreases in all three lignin monomers (H-, G-, and S-lignin) [14].

The decrease in total lignin content as well as lignin composition in *bmr* mutant sorghum lines resulted in an increase in metabolizable energy content in the forage, and thereby in vitro organic matter digestibility for livestock [13, 15, 16]. Therefore, the *bmr* mutation of sorghum can also be a promising feedstock for biofuel and biochemical production. However, our previous study [17] showed that biomass with lower lignin content does not necessarily have better bioethanol production efficiency. This could be due to the presence of a stronger lignin structure (more carbon-carbon inter-lignin linkages) in some biomass feedstocks leading to the formation of more recalcitrant biomass even in decreased total lignin content [11, 12]. In addition, the effect of *bmr* mutation to improve biomass susceptibility for digestion could be

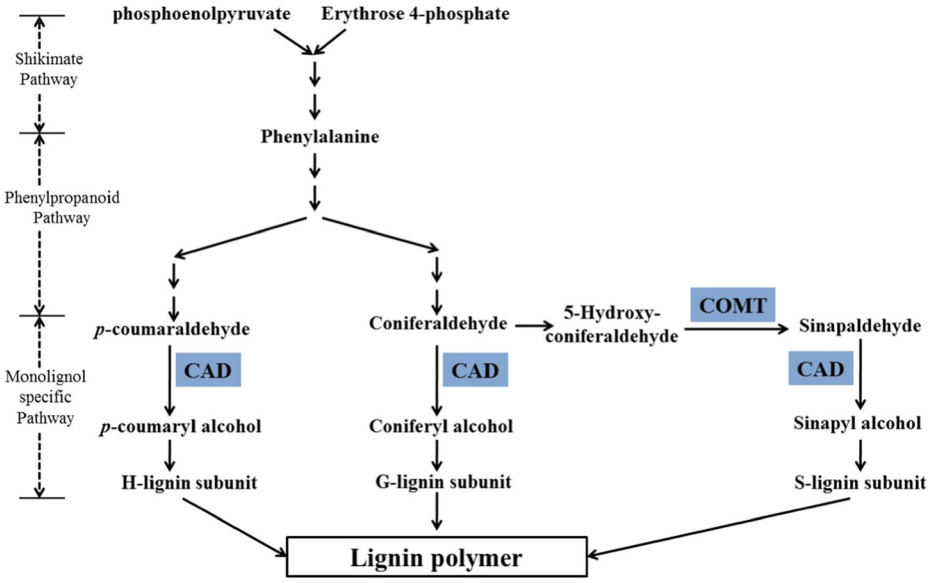

Fig. 2 The main monolignol biosynthetic pathways. *COMT* caffeic acid *O*-methyltransferase, *CAD* cinnamyl alcohol dehydrogenase [14]

attributed to the genetic background of the plant and the environment in which the plant is cultivated [15, 16, 18]. Therefore, selection of an optimal genotype for the *bmr* mutation is vital to develop a superior feedstock for biofuel and biochemical production. Besides, the altered lignin biosynthesis pathways in engineered plants frequently result in dwarfing, thereby leading to an unacceptable biomass yield penalty [19]. Studies comparing biofuel and biochemical production efficiency from *bmr* sorghum mutants and their parent lines are limited; till date, no work has been done yet for platform chemical biosynthesis, such as 2,3-butanediol. A number of bacteria, including *Klebsiella*, *Enterobacter*, and *Bacilli* genera, produce 2,3-butanediol from different sugar sources [4–7]. Most of the robust 2,3-butanediol-producing organisms reported so far, such as *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Serratia marcescens*, belong to biosafety level 2 (pathogenic) [20, 21]. The non-pathogenic (biosafety level 1) 2,3-butanediol producers such as *Paenibacillus polymyxa*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are very low efficient compared to biosafety level 2 organism [4, 20]. Recently, some strains of *B. licheniformis* such as *B. licheniformis* DSM 8785 are reported as promising non-pathogenic bacterial strains for 2,3-butanediol production using synthetic glucose medium [4, 21]; however, this bacterial strain has not been evaluated so far for 2,3-butanediol production from the lignocellulosic biomass-derived sugars, containing glucose, xylose, and other monomer sugars.

In this study, three sorghum backgrounds (Atlas, Early Hegari [EH], and Kansas Collier [KC]) and two *bmr* mutants (*bmr*6 and *bmr*12) of each line were evaluated and compared for agronomic traits, and bioprocessing efficiency to produce 2,3-butanediol from stover using *B. licheniformis* DSM 8785. The objective of this study was to test the hypothesis that the *bmr* mutation significantly changes sorghum biomass composition and leads to better-quality feedstock for 2,3-butanediol production using a robust microbial culture *B. licheniformis* DSM 8785.

Material and Methods

Sorghum Cultivation and Field Study

The forage/grain sorghum lines Atlas, Early Hegari (EH), and Kansas Collier (KC) were introgressed with two *bmr* alleles (*bmr*6 and *bmr*12) at USDA-ARS, Lincoln, Nebraska, USA [18]. The wild-type and *bmr* allele introgressed lines were evaluated in a randomized complete block design (RCBD) with three replications during post-rainy season (October) in 2011 and 2012 at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in Patancheru, Telangana, India. Each entry was planted in two rows of 2 m length with a spacing of 60 cm between rows and 15 cm between plants in a row, and fertilization was done at the rate of 64 N/28 P_2O_5 (kg ha^{-1}), 28 kg of N, and entire P_2O_5 as basal and the remaining nitrogen as top dressing at 30 days after germination. Plots were kept weed-free with chemical control and manual weeding. Timely crop protection practices were taken up, and supplemental irrigation was given whenever the precipitation was low to raise a healthy crop. The total rainfall and average daily temperature during the crop growth period were of 762 mm and 26 °C, respectively. Various agronomic traits were measured during the field study, including days to 50% flowering period, plant height (m), fresh stalk (weight of stalk at the time of harvest) yield (t/ha), stover (dry stalk) yield (t/ha), and grain yield (t/ha).

Biomass Preparation

The harvested fresh biomass was chopped (3 to 6 mm long) and dried at 60 °C in a hot air oven for a period of 6 days until the biomass moisture decreased to less than 10% (*w*/*w*). Around 200 g dried and chopped stover of all *bmr* mutants and their wild-type sorghum lines were brought from ICRISAT to Bioprocessing and Renewable Energy Laboratory, Kansas State University (KSU), Kansas. The samples were ground using a Thomas-Wiley Laboratory Mill (Model 4) fitted with a 2-mm sieve. The ground biomass samples were further separated to obtain a specific cut size by sieving in a shaker (W.S. TYLER, Model RX 29, Serial 25225) fitted with two sieves with size 20 mesh (841 μm) and 80 mesh (177 μm) [22]. The size range of the biomass was chosen based on the particle size required for biomass composition analysis without further size separation [23]. Around 2 kg *bmr*12 mutant of forage sorghum (GW8528) stalk was also ground and sieved to get the same biomass size. This sorghum line was grown at the field plot of the Kansas State University Department of Biological and Agricultural Engineering, and the biomass sample was used to optimize the pretreatment processes.

Biomass Pretreatment

The pretreatment process was first optimized to maximize sugar release from biomass. Preliminary experiments were carried out to compare acid pretreatment using 1% (*v*/*v*) sulfuric acid and 10% solid loading at 140 °C for 40 min, and alkali pretreatment using 1% (*w*/*v*) sodium hydroxide (NaOH) and 10% solid loading at 121 °C for 30 min. The results showed that alkali-pretreated biomass released almost three times more total sugars during enzymatic hydrolysis than acid-pretreated biomass (data not shown here). Then, optimum NaOH concentration for biomass pretreatment was determined by evaluating five different concentrations of NaOH solutions, 0.5, 0.75, 1.0, 1.25, and 1.5% (*w*/*v*). A 10-g ground biomass sample was mixed with 100 ml alkali solution for each concentration in a 500-ml Erlenmeyer flask and autoclaved at 121 °C for 30 min. The biomass slurry was then filtered using a 200-mesh (74 μm) sieve. Approximately 15 ml filtrate was collected to measure sugars and inhibitors produced during pretreatment, and the solid residue was washed with excess distilled water until the filtrate was clear and neutral to litmus paper. The pretreated samples were then dried overnight at 45 °C and hydrolyzed as explained in the following section. The released sugars were measured to determine the optimum alkali concentration for pretreatment. Finally, the same process was followed for the pretreatment of all sorghum samples using optimized alkali concentration.

Enzymatic Hydrolysis of Pretreated Biomass

The preliminary study was done on dose response-replacement test as recommended by the enzyme producer (Novozymes, Inc., Franklinton, NC, USA). The results showed that a total dosage of CTec2 and HTec2 released more monomer sugars from pretreated biomass compared to an equal dosage of CTec2 only (data not shown here). Therefore, the enzyme loading optimized in our previous study [22] was used in this study using both enzymes: CTec2 5.4% (*w*/*w*) of biomass (or 4.2 FPU per gram biomass) and HTec2 one tenth of CTec2 by weight (0.6%, *w*/*w*, of biomass). Pretreated biomass (2 g) was mixed with 40 ml citrate buffer (4.8 pH and 0.05 M) in a 125-ml conical flask with a screw cap. Cellic CTec2 and Cellic HTec2 enzymes were added at the rate of 5.4 and 0.6% (*w*/*w*), respectively, of biomass and incubated

in a shaker at 50 °C and 150 rpm. Five hundred-milliliter samples were drawn at different time intervals from each flask to measure released monomer sugars. Hydrolyzates were separated by centrifuging the biomass slurry at 13000 rpm (maximum g-force 20,400×*g*) for 15 min.

Fermentation of Hydrolyzates

The *B. licheniformis* DMS8785 culture was procured from Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The culture was revived in nutrient broth and preserved in 15% glycerol media comprising 0.5 ml each revived culture and 30% glycerol in 1.5-ml culture tubes. Initial experiments were carried out for growth curve study of the microbial culture to determine the optimum time for inoculum preparation; the results showed that 6 to 7 h inoculum preparation time was optimum (unpublished data). We also evaluated the sugar consumption efficiency in mixed sugar medium, containing synthetic glucose and xylose in 2:1 ratio; the results showed that the organism utilized xylose after all glucose in the medium was used up (unpublished data). To prepare inoculum, 1 ml stock culture was aseptically added into 80 ml sterilized (121 °C for 15 min) nutrient broth in a 1000-ml Erlenmeyer flask and incubated at 30 °C and 200 rpm. For fermentation, a 2-ml filter-sterilized supplement nutrient was aseptically added into the 16-ml sterilized (121 °C for 15 min) biomass hydrolyzates in 125-ml flasks. The supplement nutrient solution was prepared in the concentration so that when 2 ml of concentrated solution was added to make 20 ml total fermentation media, the final concentration would be as follows. Micronutrients (per liter): 5 g yeast extract, 5 g Bacto Tryptone, 7 g dipotassium phosphate, 5.5 g monopotassium phosphate, 1 g ammonium sulfate, 0.25 g magnesium sulfate heptahydrate, 0.12 g sodium molybdate dihydrate, 0.021 g calcium chloride dihydrate, 0.029 g cobalt nitrate hexahydrate, and 0.039 g ferrous ammonium sulfate hexahydrate. Trace elements (per liter): 0.002 g nicotinic acid, 0.000172 g sodium selenite, 0.000037 g nickel(II) chloride hexahydrate, 0.005 g manganese chloride tetrahydrate, 0.001 g boric acid, 0.000172 g aluminum potassium sulfate dodecahydrate, 0.00001 g^1 copper(II) chloride dihydrate, and 0.00554 g disodium ethylenediaminetetraacetate [21]. Control flasks were also prepared by using the same volume of synthetic medium containing 25 g/l glucose and 12 g/l xylose instead of biomass hydrolyzates. Freshly prepared 2 ml inoculum was added into each flask and incubated in a shaker at 30 °C and 200 rpm for 20 h. Samples were collected at 0, 12, and 20 h of fermentation to measure products and residual sugars.

Analytical Procedures

Biomass moisture was determined using an electric moisture meter (IR35M-00015V1, Denver Instrument GmbH, Goettingen, Germany). Extractives, lignin, glucan, xylan, and arabinan were measured using standard protocols [23, 24]. Sugars (glucose, xylose, sucrose, fructose, and arabinose), 2,3-butanediol, glycerol, acetoin, and ethanol were measured using an HPLC instrument (Shimadzu Corporation, Japan) equipped with an LC-20AB pump, an SIL-20 AC auto sampler, an SPD-M20A photodiode array detector, and a Phenomenex RCM-Monosaccharide Ca^+ column (300 × 7.8 mm). Deionized water was used as mobile phase at a flow rate of 0.6 mL min^{-1}. The column oven and refractive index detector (RID-10A) were maintained at 80 and 65 °C, respectively. To measure hydroxymethylfurfural (HMF), furfural, acetic acid, lactic acid, and formic acid, an ROA organic acid column (300 × 7.8 mm) and both RID and photodiode array (PDA)-UV detectors were used in the same HPLC system [25].

Total phenolics were determined using a modified Folin-Ciocalteu reagent (FCR) method [26]. In brief, a 0.1-ml sample (neutralized and diluted, if required) was mixed with 5 ml FCR in a 15-ml centrifuge tube. After mixing for approximately 5 min, 3.5 ml 11.5% (*w*/*v*) sodium carbonate solution was added and mixed well. A blank was prepared using 0.1 ml deionized water instead of the sample. The mixture was incubated at 40 °C for 1 h, and absorbance was taken at 745 nm. Concentration of total phenolics was determined using a standard curve. The initial experiment showed that the standard curve using only one phenolic (gallic acid) did not work well for samples containing two or more phenolics. Therefore, a standard curve was prepared by taking several concentrations of a mixture of five different phenolics: vanillic acid, catechol, gallic acid, guaiacol, and vanillin.

All experiments were carried out in triplicates, and data were statistically analyzed for least significant difference (LSD) at 95% confidence level ($P < 0.05$) using JMP software (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Agronomic Data

Table 1 shows that days to 50% flowering in *bmr*12 mutants from EH and KC backgrounds were longer (58 and 76 days, respectively) than in wild parents (53 and 73 days, respectively). The plant height of all mutants was statistically equal to the wild parents, except KC *bmr*6, which had more plant height (2.1 m) than its parent (1.8 m). The fresh stalk yield and stover yield were significantly increased in both *bmr* mutants of KC background, but it was decreased in EH *bmr*6 and AT *bmr*12 compared to their parent lines. Atlas wild type recorded the highest fresh stalk yield and stover yield (19 and 6 t/ha, respectively) followed by Atlas and KC mutants. The grain yield of EH *bmr*6 was significantly more, and Atlas *bmr*12 was significantly less than their parent lines, but no significant change in grain yield was observed in other *bmr* mutants compared to the wild parents. A similar trend in plant height and dry biomass yield in *bmr* mutants was reported by Oliver et al. [15] and Pedersen et al. [18] for EH

Table 1 Agronomic data of different sorghum genotypes

Genotypes	50% flowering time (day)	Plant height (m)	Fresh stalk yield (t/ha)	Stover yield (t/ha)	Grain yield (t/ha)
Early Hegari (EH)	53.3 ± 1.0d	1.3 ± 0.2cd	9.9 ± 1.5c	4.4 ± 0.6b	1.1 ± 0.1c
EH *bmr*6	54.3 ± 2.0d	1.1 ± 0.1d	5.9 ± 1.3d	2.5 ± 1.6c	2.0 ± 1.1b
EH *bmr*12	58.1 ± 1.6c	1.3 ± 0.3c	10.6 ± 0.9c	4.5 ± 0.9b	1.5 ± 0.3bc
Atlas (AT)	72.9 ± 1.4b	2.0 ± 0.3ab	19.2 ± 3.8a	5.8 ± 0.5a	4.5 ± 0.6a
AT *bmr*6	74.0 ± 1.1b	2.0 ± 0.1ab	18.3 ± 0.7a	5.2 ± 0.5ab	3.9 ± 0.5a
AT *bmr*12	73.0 ± 1.5b	1.8 ± 0.1b	14.8 ± 1.5b	4.4 ± 0.5b	1.9 ± 0.4b
Kansas Collier (KC)	73.4 ± 0.9b	1.8 ± 0.1b	10.5 ± 1.0c	4.5 ± 0.4b	1.6 ± 0.3bc
KC *bmr*6	74.0 ± 0.9b	2.1 ± 0.1a	17.2 ± 1.8a	5.8 ± 0.6a	2.1 ± 0.8b
KC *bmr*12	76.1 ± 1.9a	1.8 ± 0.2b	13.7 ± 2.7b	5.8 ± 1.4a	1.8 ± 0.7bc

Data are average values of triplicate experiments ± sample standard deviation. Values with the same lowercase letters, within the same column, are not significantly different from each other at the $P < 0.05$ level

bmr brown midrib sorghum mutant

and Atlas background, but an opposite trend was observed in KC background; a similar trend was found for 50% flowering time in all three backgrounds. These results indicate that the effects of the *bmr* mutation on agronomic traits depend on the parent sorghum lines in which the mutation is introduced and the environment in which the plant is cultivated. Therefore, each sorghum line must be evaluated separately to select the promising *bmr* sorghum lines that lead to increase in stover yield without significant decrease in grain yield and increase in flowering time [27].

Composition of Raw Biomass

The composition of raw biomass samples was determined for total lignin, carbohydrate polymer (glucan, xylan, and arabinan), and extractive (sum of water-soluble and alcohol-soluble extractives) contents. Table 2 shows that lignin content in *bmr* mutants was 10 to 25% lower than in parent lines, except EH *bmr*12. The greatest decrease (25%) was observed in EH *bmr*6 and Atlas *bmr*12, and the smallest decrease (10%) was in Atlas *bmr*6. EH *bmr*12 had 12% more lignin than its parent line, perhaps because of excessive production of the 5-hydroxy guaiacyl lignin monomer, which surpassed the decrease in the syringyl lignin monomer with introgression of the *bmr*12 gene in EH; *bmr12* mutation decreases activities of COMT enzymes, leading to a decrease in syringyl lignin monomer and an elevation of 5-hydroxy guaiacyl lignin monomer synthesis in plants [14]. Higher total lignin content in EH *bmr*12 is consistent with the study of Srinivasa Rao et al. [16] at ICRISAT, India, which showed that EH *bmr*12 mutant had 24 to 54% more acid detergent lignin (ADL) than other *bmr* mutants in EH, Atlas, and KC backgrounds. On the other hand, our result on EH *bmr*12 is inconsistent with the study by Oliver et al. [15] at Nebraska, USA, for EH line and its *bmr* mutants. These differences could be due to the genotype × environment (GXE) interaction as we tested temperate materials in tropical conditions. In addition, a significant variation in relative lignin content was reported in literature for the same *bmr* lines grown in the same area at different times. For example, a study by Oliver et al. [15] in 2002 and 2003 showed that ADL content of Atlas *bmr*12 is 9% less than Atlas *bmr*6 whereas Klason lignin was 2% more in a study by Dien et al. [13] in 2005; both studies were carried out at Nebraska, USA. This

Table 2 Raw biomass composition of different sorghum genotypes

Genotypes	Composition (%, *w*/w)				
	Glucan	Xylan	Arabinan	Lignin	Extractives
Early Hegari (EH)	25.4 ± 0.5cd	15.4 ± 0.4b	2.0 ± 0.6a	12.5 ± 0.7cd	35.4 ± 1.1b
EH *bmr*6	25.3 ± 1.1cd	13.8 ± 1.3d	1.9 ± 0.8a	9.3 ± 0.5g	39.7 ± 0.3a
EH *bmr*12	27.1 ± 1.3ab	18.0 ± 0.9a	2.0 ± 0.1a	13.9 ± 0.1ab	22.8 ± 0.9d
Atlas (AT)	28.5 ± 0.4 a	15.8 ± 0.7b	2.0 ± 0.7a	14.3 ± 1.3a	26.8 ± 2.8c
AT *bmr*6	26.2 ± 0.6bc	13.7 ± 0.5d	2.2 ± 0.3a	12.9 ± 0.9bc	36.1 ± 1.5b
AT *bmr*12	25.0 ± 0.8cd	15.2 ± 0.6bc	2.1 ± 0.6a	10.8 ± 0.1ef	35.1 ± 2.4b
Kansas Collier (KC)	24.5 ± 0.9de	15.2 ± 0.4bc	1.5 ± 0.4a	11.5 ± 0.4de	33.8 ± 0.2b
KC *bmr*6	21.8 ± 1.6f	14.1 ± 0.5cd	1.4 ± 0.2a	9.2 ± 0.4g	38.9 ± 1.1a
KC *bmr*12	23.2 ± 0.6ef	15.8 ± 1.0b	1.5 ± 0.1a	9.6 ± 0.7fg	35.9 ± 0.1b

Data are average values of triplicate experiments ± sample standard deviation. Values with the same lowercase letters, within the same column, are not significantly different from each other at the $P < 0.05$ level

bmr brown midrib sorghum mutant

indicated that change in lignin content in *bmr* mutant depends on both genetic background of the sorghum line as well as the environment.

Glucan and xylan content decreased in all *bmr* mutants compared with their parents, except EH *bmr*12, but change in arabinan content was not statistically significant at the 95% confidence level. Total carbohydrate polymers decreased by 4% in EH *bmr*6, by 9% in both *bmr* mutants of Atlas, and by 9 and 2% in KC *bmr*6 and KC *bmr*12, respectively, than their parent lines. But the carbohydrate polymers increased by 10% in EH *bmr*12 than its parent line. Total extractive content increased by 6 to 35% in all *bmr* mutants compared with their parents, except EH *bmr*12, in which a decrease by 36% was observed. The highest increase in extractive content (35%) compared with the parent line was observed in Atlas *bmr*6, followed by a 31% increase in Atlas *bmr*12, a 15% increase in KC *bmr*6, a 12% increase in EH *bmr*6, and a 6% increase in KC *bmr*12.

Figure 3 shows that water-soluble extractives accounted for almost 90% of total extractives, and the remaining was alcohol-soluble extractives. The water-soluble extractives include non-structural sugars, nitrogenous material, and other inorganic materials. Alcohol-soluble extractives include waxes, chlorophylls, and other minor components [24]. Sucrose, glucose, and fructose were the major non-structural sugars in water-soluble extractives, accounting for more than 50% of total extractives except in EH *bmr*12. The highest amount of non-structural sugars (28% of biomass) was found in KC *bmr*6, and the lowest (3% of biomass) in EH *bmr*12. The *bmr* mutation led to an increase in non-structural sugars by 86 and 93% compared with the parent in Atlas *bmr*6 and Atlas *bmr*12, respectively. Similarly, KC *bmr*12 and KC *bmr*6 had 58 and 66%, respectively, more non-structural sugars than the parent plant. EH *bmr*6 had 24%

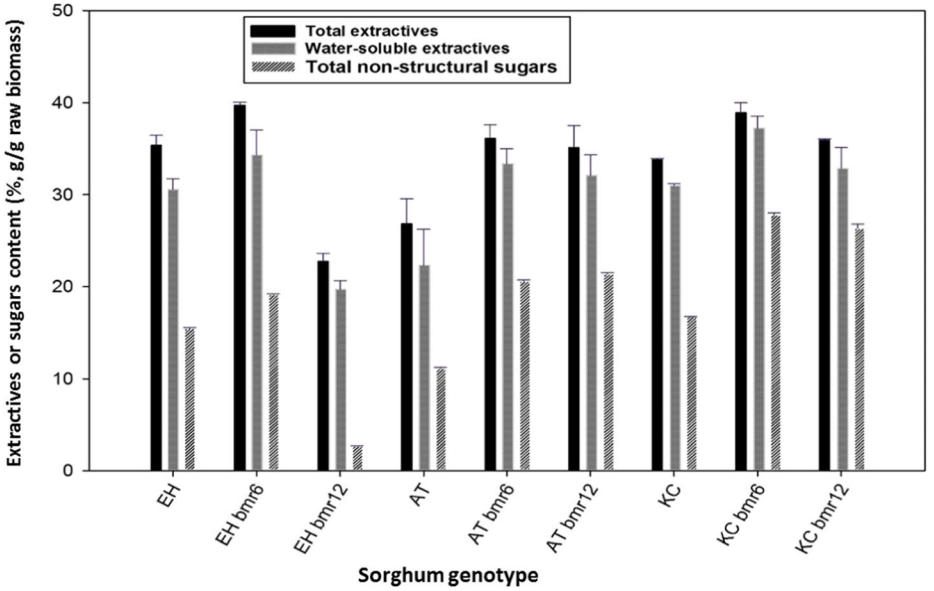

Fig. 3 Biomass extractives. *EH* Early Hegari, *AT* Atlas, *KC* Kansas Collier, *bmr* brown midrib sorghum mutant. Total extractives is the sum of water-soluble extractives and alcohol (95% ethanol)-soluble extractives. Non-structural sugars are the sugars extracted from biomass in water. All the extractions were done using Soxhlet extraction set. Data are average values of triplicate experiments, and error bars represent sample standard deviation

more non-structural sugars than its parents; however, EH *bmr*12 led to a decrease in non-structural sugars by 82%.

Biomass composition results showed that the *bmr* mutation led to significant alterations of biomass composition, and the effect considerably depends on the parent line in which the loci resides. In addition, sorghum stover contains a huge amount of non-structural sugars, which is further elevated in *bmr* mutants. Achieving additional benefits from *bmr* mutation requires these non-structural sugars to be extracted with hot water before biomass pretreatment because the presence of a number of inhibitory compounds for fermenting microbes renders the sugars released in pretreatment slurry useful as fermentable sugars. Alternatively, juice can be extracted from fresh stalks immediately after harvesting crops to recover a maximum proportion of non-structural sugars, and later combined with biomass hydrolyzates for biofuel production.

Optimization of Biomass Pretreatment

Effectiveness of dilute acid and that of alkali at the same concentration were first compared for the pretreatment of *bmr* sorghum. The results (not shown here) showed that alkali pretreatment led to significantly higher sugar yield during enzymatic hydrolysis than acid pretreatment. As a consequence, the alkali (sodium hydroxide, NaOH) concentration was optimized for biomass pretreatment at 121 °C for 30 min with 10% solid loading. Figure 4 shows that increasing NaOH concentration for pretreatment from 0.5 to 1.5% (*w*/*v*) resulted in a gradual decrease in

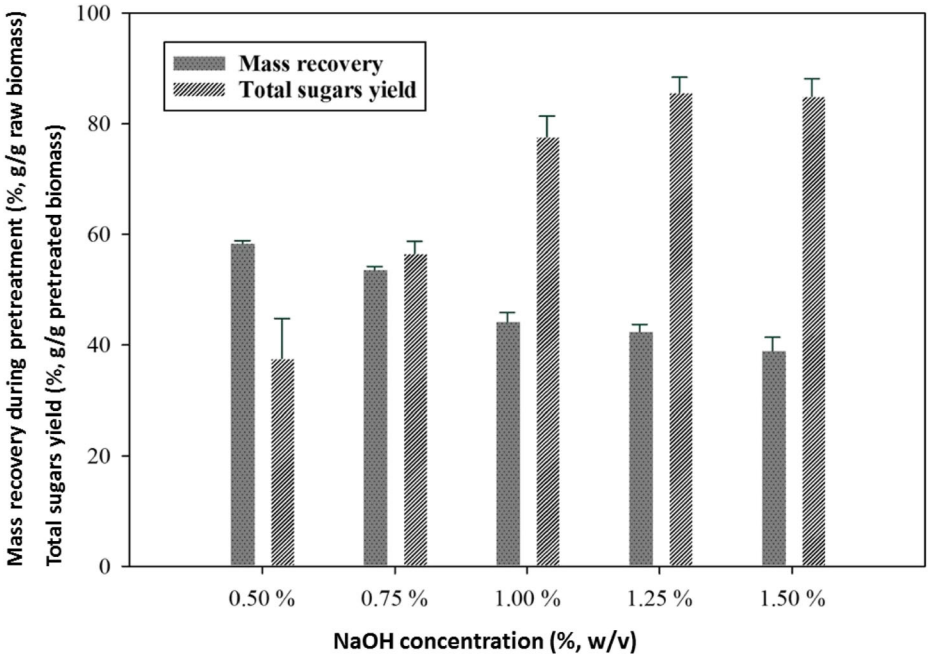

Fig. 4 Optimization of sodium hydroxide (NaOH) concentration for *bmr* sorghum pretreatment. Pretreatment was carried out at 121 °C for 30 min using 0.5 to 1.5% (*w*/*v*) NaOH with 10% (*w*/*v*) solid loading, followed by hydrolysis at 50 °C for 48 h with 5% (*w*/*v*) solid loading in citrate buffer (pH 4.8 and 0.05 M) using enzyme loading of 6% (*w*/*w*) of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation

solid mass recovery after pretreatment from 58 to 39%. On the other hand, increasing NaOH concentration from 0.5 to 1.25% increased sugar release during enzymatic hydrolysis from 38 to 86% of pretreated biomass, but increases beyond 1.25% NaOH concentration conferred no additional benefit. Based on raw biomass weight, the maximum total sugar yield (36 g/g raw biomass) was obtained from pretreated biomass with 1.25% NaOH, which was taken as the optimum alkali concentration for the comparative evaluation of various sorghum genotypes.

Mass Recovery After Pretreatment and Composition of Pretreated Biomass

Table 3 shows that the solid mass recovery during alkali pretreatment varied from 39% (KC *bmr*6) to 55% (EH *bmr*12). The KC *bmr* mutants had significantly lower mass recovery than their parent, but it was statistically equal to Atlas *bmr* mutants and their parent. Glucan, xylan, and arabinan content in pretreated biomass (Table 3) were almost double that in raw biomass (Table 2) for all sorghum lines because of the removal of a large proportion of extractives and lignin during pretreatment. However, the percentage carbohydrate increase due to pretreatment was not equal in these sorghum lines, which indicates that loss of biomass components during pretreatment varied significantly among sorghum lines. For example, EH *bmr*12 had 10% more total carbohydrate than its parent in raw biomass but 3% less than its parent in pretreated biomass; opposite results were observed for Atlas *bmr*6 and its parent line. Decreases in lignin content in pretreated biomass compared with raw biomass seemed very low or even negative in some samples because most of the biomass samples contained more than 30% extractives, which were almost completely removed during pretreatment. This led to increased lignin content in some pretreated biomass despite partial delignification. For example, lignin content in EH *bmr*6 was 9.3 and 12.9% in raw and pretreated samples, respectively, even though 37% of raw biomass lignin was removed during pretreatment. Similarly, lignin content in both raw and pretreated KC *bmr*6 was 9.2%, whereas 62% of raw biomass lignin was removed during pretreatment. Maximum delignification (around 70% of raw biomass lignin) was observed in EH, Atlas, and Atlas *bmr*6. Pretreated biomass had almost five times less total extractive

Table 3 Mass recovery after pretreatment and composition of pretreated biomass

Sorghum genotype	Mass recovery (%, g/g) biomass)	Biomass composition (%, g/g biomass)				
		Glucan	Xylan	Arabinan	Lignin	Extractive
Early Hegari (EH)	44.6 ± 2.0b	48.8 ± 2.2abc	29.8 ± 1.2a	2.7 ± 0.2abcd	8.0 ± 0.2g	6.2 ± 0.6e
EH *bmr*6	45.2 ± 1.0b	45.1 ± 2.0d	25.9 ± 0.3c	2.1 ± 0.1d	12.9 ± 0.3a	7.7 ± 0.1cd
EH *bmr*12	55.3 ± 0.6a	46.9 ± 1.2bcd	29.3 ± 1.9ab	2.4 ± 0.1abcd	8.5 ± 0.3fg	7.5 ± 0.7d
Atlas (AT)	44.7 ± 2.4b	49.4 ± 1.9ab	27.1 ± 0.6bc	2.8 ± 0.6abc	9.7 ± 0.4cd	7.1 ± 0.5d
AT *bmr*6	42.8 ± 0.8bc	51.2 ± 0.9a	27.3 ± 1.1bc	3.0 ± 0.8a	8.5 ± 0.3fg	7.4 ± 0.4d
AT *bmr*12	45.0 ± 2.4b	46.4 ± 1.0cd	28.3 ± 1.7abc	2.5 ± 0.5abcd	11.7 ± 0.7b	8.5 ± 0.6bc
Kansas Collier (KC)	44.4 ± 1.2b	47.1 ± 1.0bcd	28.8 ± 1.3ab	2.9 ± 0.4ab	10.2 ± 0.5c	9.2 ± 0.4b
KC *bmr*6	38.7 ± 1.8d	46.2 ± 0.4d	27.9 ± 2.7abc	2.3 ± 0.1bcd	9.2 ± 0.6de	11.0 ± 0.4a
KC *bmr*12	41.5 ± 1.3c	46.8 ± 2.1bcd	28.7 ± 0.9ab	2.2 ± 0.1cd	8.8 ± 0.2ef	11.1 ± 0.8a

Data are average values of triplicate experiments ± sample standard deviation. Values with the same lowercase letters, within the same column, are not significantly different from each other at the $P < 0.05$ level

bmr brown midrib sorghum mutant

content than raw biomass in all sorghum lines, indicating that 80 to 90% of extractives were removed during alkali pretreatment. In addition, Fig. 5 shows that average glucan recovery during pretreatment varied from 78 to 94%, and xylan recovery varied from 76 to 90%; however, the differences of glucan and xylan recoveries between *bmr* mutants and their parents were not statistically significant, except glucan in EH *bmr*12. These data indicated that the major factor for the biomass composition changes before and after alkali pretreatment was the removal of extractives and lignin during pretreatment along with a small portion of carbohydrate loss.

Sugars Loss and Inhibitory Compounds Produced During Pretreatment

During biomass pretreatment, hemicellulose is partially hydrolyzed to monomer sugars, and several toxic compounds, including phenolics, acetic acid, formic acid, hydroxymethylfurfural (HMF), and furfural, are produced as a result of depolymerization of lignin and degradation of released sugars. These compounds are considered toxins because they inhibit sugar-fermenting microbes [28]. Table 4 shows that total sugar release during pretreatment was 2.5 to 17.3% of raw biomass, which is close to the non-structural sugar content (Fig. 3). This result indicates that hemicellulose was not hydrolyzed significantly during pretreatment. Higher acetic acid production (3.1 to 4.5% of raw biomass) compared with phenolics (2.6 to 3.6% of raw biomass) and minimum hydrolysis of hemicellulose indicates that sorghum lignin is extensively acylated; biomass lignin is partially acylated at γ-carbon of the lignin monomer [11, 29]. Formic acid was produced at 0.4 to 0.7% of raw biomass. Degradation of xylose and arabinose produces furfural, and further degradation of furfural produces formic acid [30]. HMF and

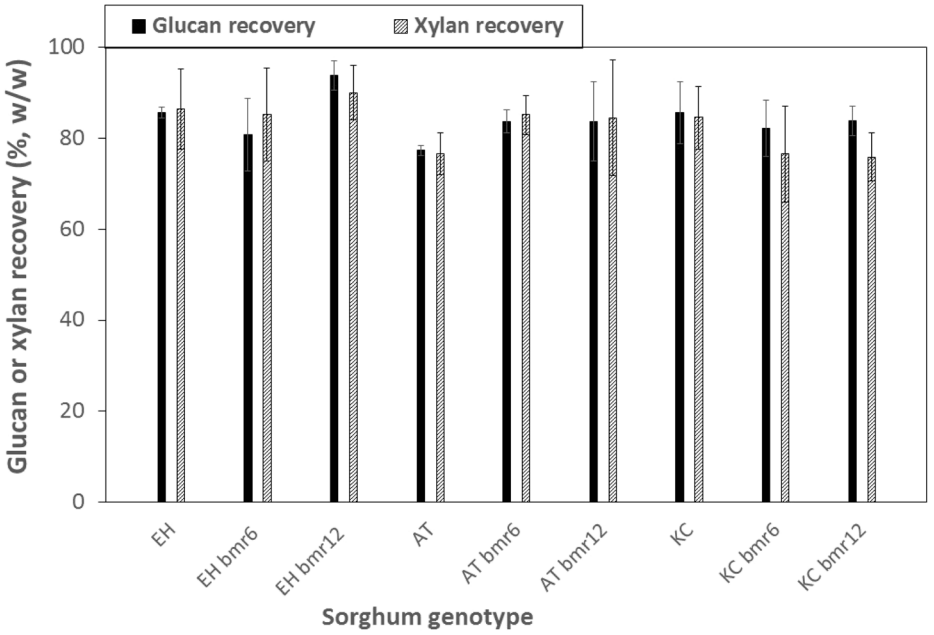

Fig. 5 Glucan and xylan recoveries during alkali pretreatment of biomass. *EH* Early Hegari, *AT* Atlas, *KC* Kansas Collier, *bmr* brown midrib sorghum mutant. Pretreatment was carried out at 121 °C for 30 min using 1.25% (*w*/*v*) sodium hydroxide solution with 10% (*w*/*v*) solid loading. Data are average values of triplicate experiments, and error bars represent sample standard deviation

Table 4 Sugars and inhibitory compounds released during pretreatment

Phenotypes	Released compounds (%, g/g biomass)			
	Total sugars	Phenolics	Acetic acid	Formic acid
Early Hegari (EH)	16.6 ± 0.8a	3.6 ± 0.3a	4.5 ± 0.3a	0.68 ± 0.03ab
EH *bmr*6	14.7 ± 2.0a	3.3 ± 0.4ab	3.2 ± 0.4b	0.51 ± 0.04cd
EH *bmr*12	2.5 ± 0.1c	3.6 ± 0.1a	4.1 ± 0.0ab	0.42 ± 0.01d
Atlas (AT)	6.6 ± 0.8b	3.3 ± 0.2ab	3.1 ± 0.4b	0.58 ± 0.06bc
AT *bmr*6	14.4 ± 2.7a	3.2 ± 0.4ab	3.5 ± 0.6b	0.68 ± 0.10ab
AT *bmr*12	10.3 ± 4.1b	3.0 ± 0.3bc	3.3 ± 1.4b	0.67 ± 0.22b
Kansas Collier (KC)	8.3 ± 0.6b	3.0 ± 0.1bc	4.6 ± 0.4a	0.85 ± 0.07a
KC *bmr*6	17.3 ± 3.1a	2.6 ± 0.2c	3.4 ± 0.6b	0.69 ± 0.11ab
KC *bmr*12	15.6 ± 1.5a	2.6 ± 0.2c	3.9 ± 0.3ab	0.71 ± 0.02ab

Data are average values of triplicate experiments ± sample standard deviation. Values with the same lowercase letters, within the same column, are not significantly different from each other at the $P < 0.05$ level

bmr brown midrib sorghum mutant

furfural were also measured in all biomass samples, but their values were very low (less than 0.01% of raw biomass), and hence are not reported here. These low values show that 1.25% NaOH pretreatment did not significantly degrade sugars to HMF, and the small amount of furfural produced during this process almost completely further degraded to formic acid.

Hydrolysis of Pretreated Biomass

Total sugar yield based on raw biomass was 19, 16, and 14% more in *bmr*12 of EH, Atlas, and KC, respectively, than in their parents, whereas the *bmr*6 mutants did not yield a significantly higher amount of total sugars than their parents (Fig. 6). Based on total carbohydrate content in raw biomass, total sugar yield was 8 to 27% more in *bmr* mutants than in their parents. In EH background, total sugar yield in *bmr*6 based on total carbohydrate in raw biomass was significantly more than *bmr*12 at the 95% confidence level, but the opposite was true based on total raw biomass weight, which was due to the higher carbohydrate loss in *bmr*6 during the pretreatment process. In Atlas background, *bmr*12 had significantly higher total sugar yield than *bmr*6 based on both raw biomass weight as well as total carbohydrate content. In KC background, both *bmr* mutants had statistically equal total sugar yield. The results also showed that glucose and xylose released during hydrolysis varied from 64 to 68% and 30 to 33% of total sugars, respectively, whereas arabinose released was 3% of total sugars in all samples. However, the difference in the proportion of glucose and xylose released during hydrolysis among these samples was not statistically significant at the 95% confidence level. These results indicated that *bmr* mutation led to an increase in the hydrolysis efficiency of biomass; however, the effects varied significantly among biomass types as well as *bmr* types.

Fermentation of Biomass Hydrolyzates

Figure 7 shows that 2,3-butanediol yield per gram of sugars consumed during fermentation was not significantly different among biomass hydrolyzates and the control at a 95% confidence level. At 12 h of fermentation, almost all glucose and arabinose and around 50% of xylose were metabolized by the bacterial culture, *B. licheniformis* DSM 8785. When the fermentation period was extended for 20 h, all monomer sugars were completely metabolized.

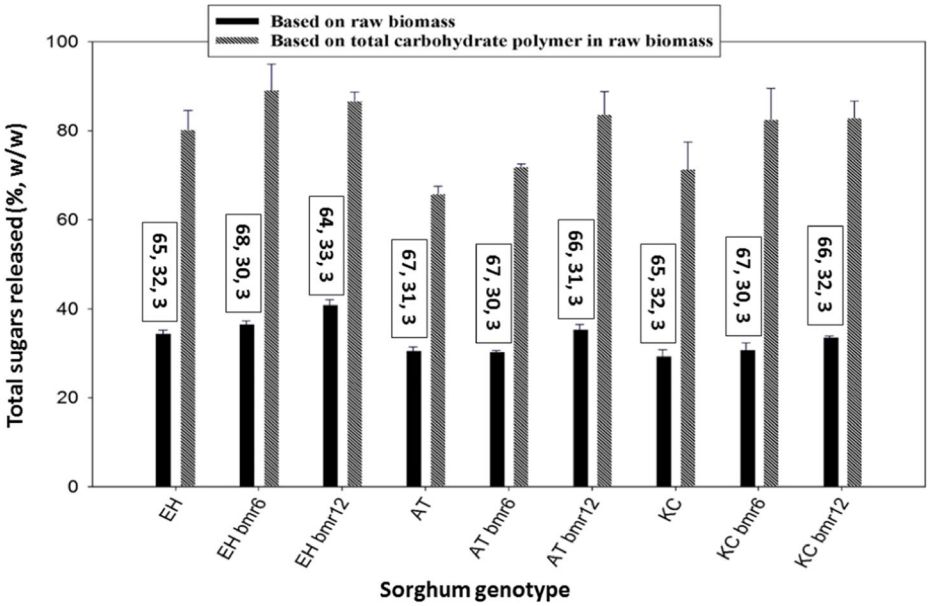

Fig. 6 Total sugars released during hydrolysis of pretreated biomass. *EH* Early Hegari, *AT* Atlas, *KC* Kansas Collier, *bmr* brown midrib sorghum mutant. Pretreatment was carried out at 121 °C for 30 h with 5% (*w*/*v*) biomass loading in citrate buffer (pH 4.8 and 0.05 M) using enzyme loading of 6% (*w*/*w*) of biomass. Data are average values of triplicate experiments, and error bars represent sample standard deviation. Three numerical values above the first bar of each sorghum genotype represent the percentage (of total sugar) of glucose, xylose, and arabinose released during hydrolysis, respectively

This result indicates that quality of released sugars does not vary among sorghum lines and their *bmr* mutants, and are comparable with the quality of synthetic sugars for 2,3-butanediol production using *B. licheniformis*. Average 2,3-butanediol yield was very low (around 0.3 g per g sugars consumed), however, in all samples because of the production of a significant amount of by-products, including acetic acid, glycerol, lactic acid, and ethanol (data not reported here); the theoretical maximum yield is 0.50 g 2,3-butanediol per gram of glucose [21]. Fermentation parameters, including pH, aeration, and agitation, must be optimized to minimize by-product formation to funnel maximum carbon from sugars to 2,3-butanediol [4]. The 2,3-butanediol yield per gram of raw sorghum biomass (Fig. 7) showed the same trend to that of sugar released during hydrolysis (Fig. 6), that is, *bmr*12 had higher 2,3-butanediol yield than their parents, whereas *bmr*6 and parents had statistically equal 2,3-butanediol yield.

Overall Mass Balance From Sorghum Stover to Fermentable Sugars

Overall mass balances from raw biomass to total fermentable sugars (sum of total sugars released by hydrolysis of pretreated biomass and non-structural sugars obtained from water extraction of raw biomass) are shown in Fig. 8. The EH *bmr*12 mutant yielded the highest total sugars (0.41 g/g raw biomass) from hydrolysis; however, it had the lowest total fermentable sugars because of a very low amount of non-structural sugars (0.03 g/g raw biomass). The total fermentable sugar yield in the EH *bmr*6 mutant is 12% more than in its parent, but it was 12% less than the parent line for the EH *bmr*12 mutant. In addition, the stover yield in EH *bmr6* mutants was almost half of its parent line (Table 1), indicating that *bmr* mutation of EH

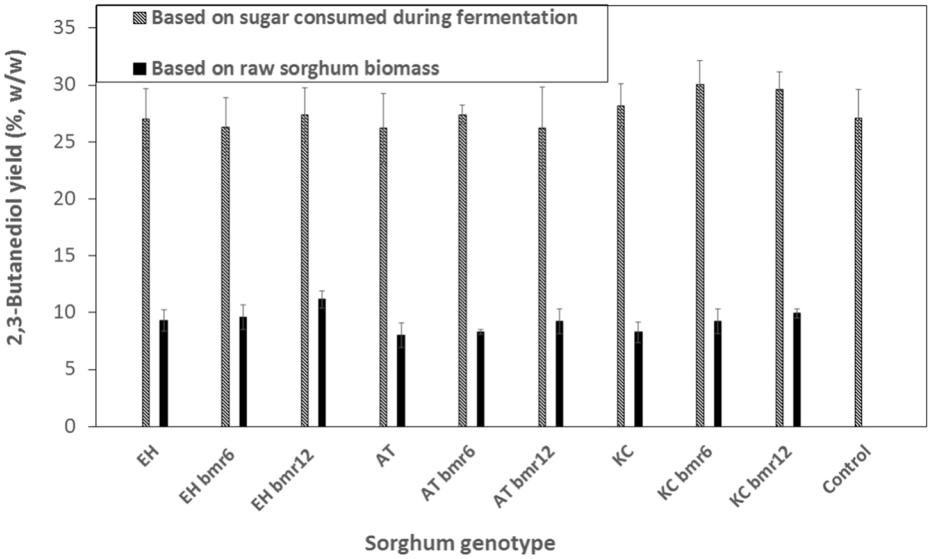

Fig. 7 2,3-Butanediol fermentation from biomass hydrolyzates. *EH* Early Hegari, *AT* Atlas, *KC* Kansas Collier, *bmr* brown midrib sorghum mutant, control = synthetic sugar solution with similar concentration of hydrolyzates. Fermentation was carried out at 30 °C and 200 rpm for 12 h using *Bacillus licheniformis* DSM 8785. Data are average values of triplicate experiments, and error bars represent sample standard deviation

sorghum is not a good approach to produce feedstock for biofuel production. On the other hand, total fermentable sugars in Atlas *bmr*12 and KC *bmr*12 mutants were 36 and 30%, respectively, more than their parents; Atlas *bmr*6 and KC *bmr*6 mutants yielded 22 and 27%, respectively, more than the parents. Stover yield in KC *bmr*6 and KC *bmr*12 mutants had 64 and 31%, respectively, more than their parent line (Table 1). In addition, the grain yield in both *bmr* mutants in KC background was not significantly different from the wild-type parent line. The stover and grain yields in Atlas *bmr6* mutant are statistically equal to its wild-type parents, but not Atlas *bmr*12. Overall, introgression of the *bmr*6 gene in the KC line led to the most promising feedstock among the tested sorghum lines for second-generation biofuel and biochemical production without affecting grain yield, followed by KC *bmr*12 and Atlas *bmr*6. Introgression of the *bmr*12 gene in Atlas background also led to improved feedstock quality for biofuel and chemical production, but grain yield is penalized. The *bmr* mutation in EH background adversely affected the feedstock quality.

Conclusions

The *bmr* mutant lines significantly affected their flowering time, grain, and stover yields as well as composition of biomass as compared to their wild-type counterparts. These effects led to improvement on quality of biomass for platform chemicals, like 2,3-butanediol production in some lines and *bmr* types, while an adverse effect was observed in others. Introgression of the *bmr*6 gene in the KC line led to the most promising feedstock among the tested sorghum lines for second-generation biofuel and biochemical production without affecting grain yield, followed by KC *bmr*12 and Atlas *bmr*6, but an adverse effect was observed in both *bmr* mutants in the EH line. These results indicated a significant interaction

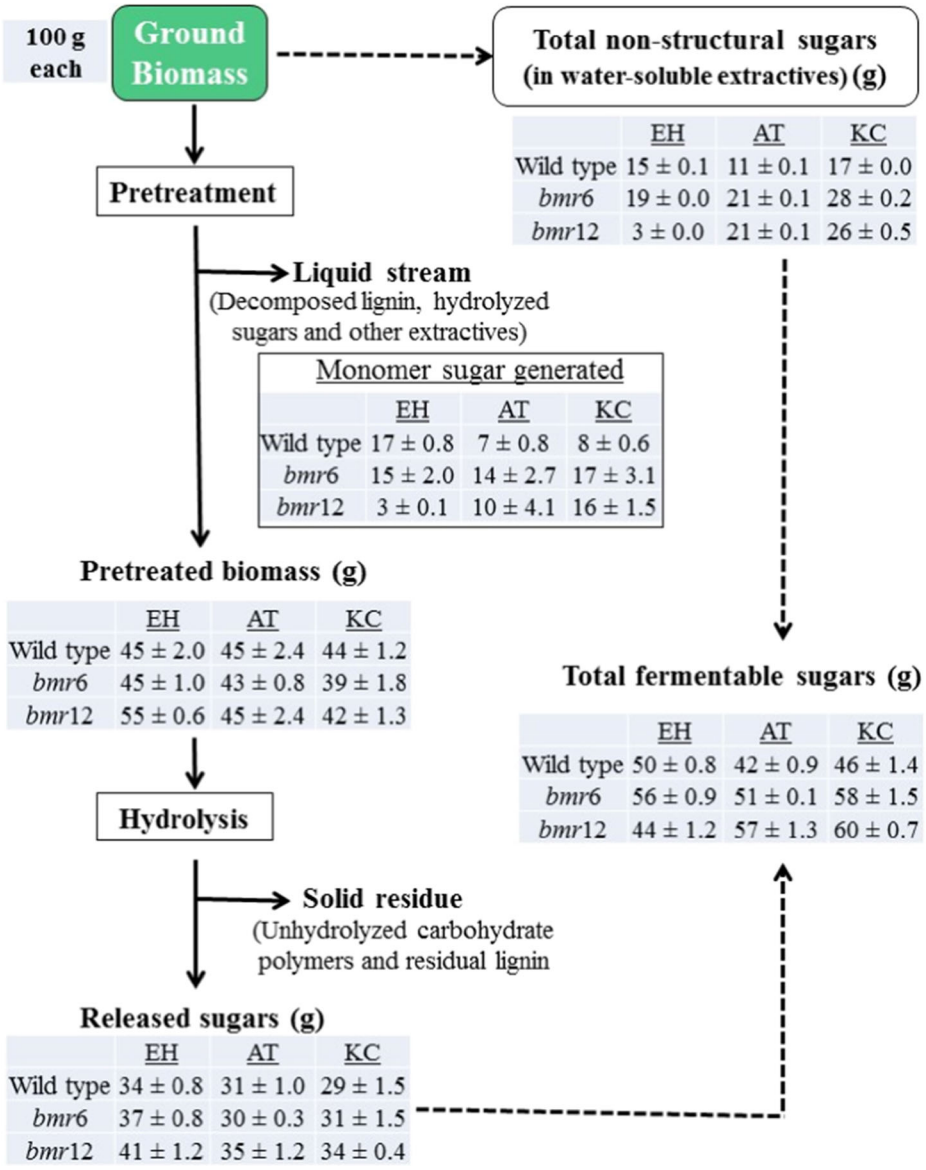

Fig. 8 Overall mass balance from sorghum stover to total fermentable sugars. *EH* Early Hegari, *AT* Atlas, *KC* Kansas Collier, *bmr* brown midrib sorghum mutant. Data are average values of triplicate experiments ± sample standard deviation

effect between the *bmr* gene and the genetic background of the sorghum lines in which the *bmr* gene is introduced. Therefore, each sorghum line must be evaluated separately to select the promising sorghum lines for biofuel and biochemical production. In addition, the quality of released sugars from alkali-pretreated biomass is unaffected by the background and *bmr* mutant, and the sugar quality was as good as the synthetic sugars for 2,3-butanediol production using a non-pathogenic culture *Bacillus licheniformis* DSM 8785.

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References

1. Guragain, Y. N., Probst, K. V., & Vadlani, P. V. (2016). Fuel alcohol production. In C. Wrigley, H. Corke, K. Seetharaman, & J. Faubion (Eds.), *Encyclopedia of food grains* (2nd ed., pp. 235–244). Oxford, Academic Press.
2. Klein-Marcuschamer, D., & Blanch, H. W. (2015). Renewable fuels from biomass: Technical hurdles and economic assessment of biological routes. *American Institute of Chemical Engineers Journal, 61*, 2689–2701.
3. Kamireddy, S. R., Li, J., Abbina, S., Berti, M., Tucker, M., & Ji, Y. (2013). Converting forage sorghum and sunn hemp into biofuels through dilute acid pretreatment. *Industrial Crops and Products, 49*, 598–609.
4. Ji, X. J., Huang, H., & Ouyang, P. K. (2011). Microbial 2,3-butanediol production: a state-of-the-art review. *Biotechnology Advances, 29*, 351–364.
5. Guragain, Y. N., Bastola, K. P., Madl, R. L., & Vadlani, P. V. (2016). Novel biomass pretreatment using alkaline organic solvents: a green approach for biomass fractionation and 2,3-butanediol production. *Bioenergy Research, 9*, 643–655.
6. Ge, L., Wu, X., Chen, J., & Wu, J. (2016). A new method for industrial production of 2, 3-butanediol. *Journal of Biomaterials and Nanobiotechnology, 2*, 335–336.
7. Li, L., Zhang, L., Li, K., Wang, Y., Gao, C., Han, B., Ma, C., & Xu, P. (2013). A newly isolated *Bacillus licheniformis* strain thermophilically produces 2, 3-butanediol, a platform and fuel bio-chemical. *Biotechnology for Biofuels, 6*, 123.
8. Solomon, B. D., Barnes, J. R., & Halvorsen, K. E. (2007). Grain and cellulosic ethanol: History, economics, and energy policy. *Biomass and Bioenergy, 31*, 416–425.
9. Guragain, Y. N., De Coninck, J., Husson, F., Durand, A., & Rakshit, S. K. (2011). Comparison of some new pretreatment methods for second generation bioethanol production from wheat straw and water hyacinth. *Bioresource Technology, 102*, 4416–4424.
10. Tomas-Pejo, E., Oliva, J., & Ballesteros, M. (2008). Realistic approach for full-scale bioethanol production from lignocellulose: a review. *Journal of Scientific & Industrial Research, 67*, 874–884.
11. Guragain, Y. N., Herrera, A. I., Vadlani, P. V., & Prakash, O. (2015). Lignins of bioenergy crops: a review. *Natural Product Communications, 10*, 201–208.
12. Studer, M. H., DeMartini, J. D., Davis, M. F., Sykes, R. W., Davison, B., Keller, M., Tuskan, G. A., & Wyman, C. E. (2011). Lignin content in natural *Populus* variants affects sugar release. *Proceedings of the National Academy of Sciences USA, 108*, 6300–6305.
13. Dien, B. S., Sarath, G., Pedersen, J. F., Sattler, S. E., Chen, H., Funnell-Harris, D. L., Nichols, N. N., & Cotta, M. A. (2009). Improved sugar conversion and ethanol yield for for-age sorghum (*Sorghum bicolor* L. Moenth) lines with reduced lignin contents. *Bioenergy Research, 2*, 153–164.
14. Sattler, S. E., Funnell-Harris, D. L., & Pedersen, J. F. (2010). Brown midrib mutations and their importance to the utilization of maize, sorghum, and pearl millet lignocellulosic tissues. *Plant Science, 178*, 229–238.
15. Oliver, A., Pedersen, J., Grant, R., Klopfenstein, T., & Jose, H. (2005). Comparative effects of the sorghum *bmr*-6 and *bmr*-12 genes: II. Grain yield, stover yield, and stover quality in grain sorghum. *CROP SCIENCE-MADISON, 45*, 2240–2245.
16. Srinivasa Rao, P., Deshpande, S., Blummel, M., Reddy, B. V., & Hash, T. (2012). Characterization of brown midrib mutants of sorghum (*Sorghum bicolor* (L.) Moench). *The European Journal of Plant Science and Biotechnology, 6*, 71–75.
17. Guragain, Y. N., Ganesh, K., Bansal, S., Sathish, R. S., Rao, N., & Vadlani, P. V. (2014). Low-lignin mutant biomass resources: effect of compositional changes on ethanol yield. *Industrial Crops and Products, 61*, 1–8.
18. Pedersen, J. F., Funnell, D. L., Toy, J. J., Oliver, A. L., & Grant, R. J. (2006). Registration of seven forage sorghum genetic stocks near-isogenic for the brown midrib genes bmr-6 and bmr-12. *Crop Science, 46*, 490–491.

19. Bonawitz, N. D., & Chapple, C. (2013). Can genetic engineering of lignin deposition be accomplished without an unacceptable yield penalty? *Current Opinion in Biotechnology, 24*, 336–343.
20. Yang, J., Kim, B., Kim, H., Kweon, Y., Lee, S., & Lee, J. (2015). Industrial production of 2,3-butanediol from the engineered *Corynebacterium glutamicum*. *Applied Biochemistry and Biotechnology, 176*, 2303–2313.
21. Jurchescu, I., Hamann, J., Zhou, X., Ortmann, T., Kuenz, A., Prüße, U., & Lang, S. (2013). Enhanced 2, 3-butanediol production in fed-batch cultures of free and immobilized *Bacillus licheniformis* DSM 8785. *Applied Microbiology and Biotechnology, 97*, 6715–6723.
22. Guragain, Y. N., Wilson, J., Staggenborg, S., McKinney, L., Wang, D., & Vadlani, P. V. (2013). Evaluation of pelleting as a pre-processing step for effective biomass deconstruction and fermentation. *Biochemical Engineering Journal, 77*, 198–207.
23. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. (2007). Determination of structural carbohydrates and lignin in biomass. *National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures (LAP) for standard biomass analysis*.
24. Sluiter, A., Ruiz R, Scarlata, C., Sluiter, J., Templeton, D. (2005). Determination of extractives in biomass. *National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedure (LAP),* 1617.
25. Guragain, Y. N., Wang, D., & Vadlani, P. V. (2016). Appropriate biorefining strategies for multiple feedstocks: critical evaluation for pretreatment methods, and hydrolysis with high solids loading. *Renewable Energy, 96*, 832–842.
26. Amendola, D., De Faveri, D., Egües, I., Serrano, L., Labidi, J., & Spigno, G. (2012). Autohydrolysis and organosolv process for recovery of hemicelluloses, phenolic compounds and lignin from grape stalks. *Bioresource Technology, 107*, 267–274.
27. Rao, P. S., Prakasham, R., Deshpande, S., & Umakanth, A. (2010). *Brown midrib sorghum: current status and potential as novel ligno-cellulosic feedstock of bioenergy* (p. 112). Saarbrücken: Lap Lambert Academic Publishing Gmbh and Co KG.
28. Yoo, J., Alavi, S., Vadlani, P., & Behnke, K. C. (2010). Soybean hulls pretreated using thermo-mechanical extrusion—hydrolysis efficiency, fermentation inhibitors, and ethanol yield. *Applied Biochemistry and Biotechnology, 166*, 576–589.
29. Martínez, Á. T., Rencoret, J., Marques, G., Gutiérrez, A., Ibarra, D., Jiménez-Barbero, J., & del Río, J. C. (2008). Monolignol acylation and lignin structure in some nonwoody plants: a 2D NMR study. *Phytochemistry, 69*, 2831–2843.
30. Jönsson, L. J., Alriksson, B., & Nilvebrant, N. (2013). Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnology for Biofuels, 6*, 16.