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Low-lignin mutant biomass resources: Effect of compositional changes on ethanol yield

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ABSTRACT

Low-lignin content *bmr* (Brown Mid-Rib)-mutant forage sorghum (*Sorghum bicolor*) grown at Riley and Doniphan counties (Kansas, USA) were compared with wild type high-lignin content forages to evaluate the effect of reduced lignin content in biomass on bioethanol production. Sorghum bagasse (*Sorghum bicolor*), wheat straw (*Triticum aestivum*), switchgrass (*Panicum virgatum*) and miscanthus (*Miscanthus giganteus*) were used as wild type forages. Ground biomass samples were pretreated with 2% sodium hydroxide at 121 °C for 30 min followed by enzymatic hydrolysis using cellulase complex and β-glucosidase at high solid loading (20%, W/V), and the sugars released were fermented using *Saccharomyces cerevisiae*. Lignin content of all types of biomass was reduced to statistically equal levels (2–4%) after alkali pretreatment irrespective of their initial lignin content. However, saccharification efficiency of the pretreated biomass samples varied significantly from each other and showed a weak relationship between saccharification efficiency and total lignin content in raw and/or pretreated biomass. These results indicated that the reduced total lignin content in *bmr*-mutant crops does not necessarily benefit bioethanol production. Therefore, number of other factors, including lignin structure, crystallinity of cellulose and type of linkages in cellulose-hemicellulose-lignin complex must be further assessed to evaluate new crop lines for bioethanol production. The Fourier transform infrared spectroscopy (FTIR) peak intensities of ester linkages, aromatic C=C linkages and glycosidic linkages for raw, pretreated and hydrolyzed biomass samples are in agreement with the variation in saccharification efficiencies among biomass types. Ethanol yield was more than 95% of the maximum possible theoretical yield based on released glucose in all types of biomass, which showed that biomass after alkali pretreatment followed by washing with water does not inhibit *S. cerevisiae*, regardless of feedstock sources.

1. Introduction

Gradual depletion of global reservoirs of fossil fuels and growing political conflict in key oil-producing countries increase the likelihood of future energy crises, especially for non-oil producing countries (Balat and Balat, 2009). Volatile gasoline prices coupled with these anticipated future threats have sparked the interest of scientists, lawmakers and the media in utilization of renewable energy sources (Hisano et al., 2009). Bioethanol, biobutanol, and other biofuels are some renewable fuels that can partially or completely replace fossil fuels and thereby help reduce global dependency on them (Hisano et al., 2009; Pfromm et al., 2010). First-generation biofuel production using sugar and starchy food materials as feedstocks has now technically matured. Governmental support for green energy production through subsidies and tax incentives has made first-generation biofuel production economically viable (Kim and Dale, 2004; Mousdale, 2008). But it is not considered a sustainable alternative because of scarcity of raw materials necessary to produce biofuels on a large enough scale to meet the huge demand for liquid transportation fuel as well as the food security threat associated with its production (Demirbas, 2009). In 2009, a study found that more than 30% of the corn harvested in United States (U.S.) would be consumed to produce 10 billion gallons of ethanol, which would reduce nation's gasoline

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consumption by only 4.8% (Dien et al., 2009). Other sources of renewable energy, such as solar, wind, hydroelectricity and wave energy combined cannot fulfill more than 4% of global future energy demand (Mousdale, 2008). Therefore, exploitation of lignocellulosic residues to produce bioethanol and other bio-based chemicals is considered a sustainable alternative to fossil fuels because of the materials' availability in large quantities throughout the world at comparatively low cost (Hisano et al., 2009). A U.S. government study estimated that domestic lignocellulosic residues are available in sufficient quantity to produce enough biofuels to substitute for 30% of domestic petroleum consumption (Dien et al., 2009).

Biofuels and bio-based chemicals production from lignocellulosic biomass on a commercial scale faces a number of challenges and is yet to become economically viable given the current price of petroleum products (Hu et al., 2008; Hendriks and Zeeman, 2009). The biofuel production process from lignocellulosic biomass consists of four major steps: pretreatment of biomass to make the carbohydrate polymers (cellulose and hemicellulose) susceptible to hydrolysis; enzymatic hydrolysis to depolymerize these polymers into monomer sugars; fermentation of these sugars to bio-based fuels and chemicals; and product recovery (Hu et al., 2008; Singh and Bishnoi, 2013). Although each step is associated with a number of challenges, the major roadblock is the lack of an efficient and cost-effective pretreatment method, which leads to high operational costs for biorefineries (Tomas-Pejo et al., 2008; Ren et al., 2009; Sousa et al., 2009; Ansanay et al., 2014). The main impediment to the development of an effective pretreatment process is a strong layer of lignin in the plant cell wall, which is responsible for the recalcitrance of lignocellulosic biomass toward hydrolysis of carbohydrate polymers (Hu et al., 2008; Zhao et al., 2009). High enzyme costs for the hydrolysis of pretreated biomass (Brijwani et al., 2010; Oberoi et al., 2011) and production of inhibitors along with sugars that negatively affect fermentation yields (Hu et al., 2008; Sousa et al., 2009; Zhao et al., 2009) are other major obstacles to exploitation of lignocellulosic residues. The latter problems are also related to the pretreatment process, and hence can be minimized if technically and economically efficient pretreatment processes are developed. Ideally, a good pretreatment process leads to requirement of minimal enzymes for hydrolysis and good-quality sugars for fermentation (Guragain et al., 2011, 2013; Li et al., 2009).

Selection of appropriate biomass feedstock is vital to cellulosic ethanol production (Anfinru et al., 2013; Monono et al., 2013; Wu et al., 2013). Theoretically, maximum bioethanol can be produced from biomass containing higher amount of carbohydrate polymers (Godin et al., 2013a). Considering total lignin content in biomass is a major obstacle to release of sugars from carbohydrate polymers, several researchers in plant molecular biology have started to engineer plant species to reduce the lignin content in biomass (Hisano et al., 2009). Unfortunately, disruptions of lignin biosynthesis in genetically engineered plants frequently results in dwarfing; dwarfism mechanisms in such plants are poorly understood and researchers have yet to achieve significant progress in avoiding it (Bonawitz and Chapple, 2013). Moreover, recent studies suggested that inverse correlation between lignin content in raw biomass and enzymatic hydrolysis yield of pretreated biomass does not always validate herbaceous biomasses (Leu and Zhu, 2013). Kamireddy et al. (2013) reported similar sugar yields from dilute acid pretreated *bmr* (Brown Mid-Rib) and non-*bmr* forage sorghum; the *bmr*-mutants contained significantly lower lignin than non-*bmr*-mutants. The dilute acid pretreatment makes cellulose accessible for cellulase enzyme by hydrolyzing hemicellulose polymer in lignocellulosic biomass whereas alkali pretreatment performs the same job by removing lignin (Leu and Zhu, 2013). However, to the best of our knowledge, comparative studies on *bmr* and non-*bmr*-mutant of forages to evaluate the effects of initial lignin content on bioethanol yields using alkali pretreatment process are limited in literature. In our study, a low-lignin *bmr*-mutant of forage sorghum stalk, a high-lignin content sorghum bagasse, wheat straw, miscanthus and swithchgrass were evaluated and compared for bioethanol production in terms of their compositional changes during alkali pretreatment, enzymatic hydrolysis yield of sugars and fermentation yield of ethanol.

2. Materials and methods

2.1. Materials

The *bmr*-mutants of forage sorghum (*Sorghum bicolor*) grown at Riley (BMR-RL) and Doniphan (BMR-DP) counties (Kansas, USA); swithchgrass (*Panicum virgatum*), miscanthus (*Miscanthus giganteus*), sorghum bagasse (*Sorghum bicolor*) and wheat straw (*Triticum aestivum*) were obtained for our research from the Department of Agronomy at Kansas State University in 2007–2008. All the samples were sun dried prior to receiving; these samples were first chopped into small pieces and stored in small gunny bags at room temperature. The biomass samples were ground with a Thomas-Wiley Laboratory Mill (Model 4) with 600-μm sieve before using for biomass processing in 2009.

Cellulase complex (NS22074) and β-glucosidase (NS50010) enzymes were donated by Novozymes, Inc. (Franklinton, NC); the enzymes were stored at 4 °C in refrigerator until used for the experiment. Active dry yeast, *Saccharomyces cerevisiae* was obtained from bioethanol production plant located in Scandia, Kansas, and stored at 4 °C in refrigerator until used for the experiment.

2.2. Pretreatment of biomass

The ground biomass samples were pretreated with an alkali method (Guragain et al., 2013) in which 30 g of biomass was mixed with 300 ml 2% (W/V) sodium hydroxide in a 1000-ml Erlenmeyer flask. Biomass slurries were then autoclaved at 121 °C for 30 min. Pretreated samples were then transferred onto the muslin cloth and washed with distilled water until the filtrate was clear and neutral to litmus paper. Residues were squeezed manually to remove excess water and divided into two portions. One portion was used for enzymatic hydrolysis, and the other was dried at 70 °C for 24 h and used for compositional analysis.

2.3. Enzymatic hydrolysis

2.3.1. Determination of enzyme activity

Cellulase complex and β-glucosidase activities were determined using the method described by Ghose (1987). Filter paper units (FPU) and endoglucanase units (EGU) were used as the units of enzyme activity for cellulase and β-glucosidase, respectively. One FPU is defined as the amount of enzyme required to release 1.0 μmol of reducing sugar equivalents per min from 1.0 cm × 6.0 cm (=50 mg) Whatman No. 1 filter paper strips at 50 °C and pH 4.8. One EGU is defined as the amount of enzyme required to release 1.0 μmol of glucose units from cellobiose per min at 50 °C and pH 4.8.

2.3.2. Hydrolysis of pretreated biomass

Pretreated biomass containing 10 g dry matter was mixed with a calculated amount of citrate buffer (50 mM, pH 4.8) in 250-ml polycarbonate screw-capped flasks so final slurry had 20% solid loading. To prevent undesirable microbial growth, 0.3% (W/V) sodium azide was added to the slurry. Cellulase complex and β-glucosidase enzymes were added at a concentration of 25 FPU/g and 31.3 EGU/g of dry pretreated biomass, respectively. The flasks were then incubated at 50 °C and 150 rpm in a shaker (Model