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Technical Communication

Impact of low lignin containing *brown midrib* sorghum mutants to harness biohydrogen production using mixed anaerobic consortia

R.S. Prakasham [a,*], P. Brahmaiah [a], D. Nagaiah [a], P. Srinivasa Rao [b], Belum V.S. Reddy [b], R. Sreenivas Rao [c], Phil J. Hobbs [c]

[a] Bioengineering and Environmental Centre, Indian Institute of Chemical Technology, Hyderabad 500 607, India
[b] International Crops Research Institute for the Semi-Arid Tropics, Patancheru 502 324, India
[c] Bioenergy Group, Rothamsted Research, North Wyke, Okehampton EX20 2SB, UK

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ABSTRACT

Three low lignin containing *bmr* 3 derivatives, namely DRT 07K1, DRT 07K6 and DRT 07K15 developed through backcrossing were used along with the parent, *bmr* 3 source mutant (IS 21888) for evaluation of biohydrogen production. Results demonstrated that biohydrogen production varied amongst *bmr* derivatives under similar fermentation conditions. Significant negative correlation was observed between lignin content and fermentative biohydrogen production. All *bmr* derivatives with lower lignin content produced higher levels of biohydrogen compared to source *bmr* 3 (IS 21888) which has more lignin content. The maximum and a minimum biohydrogen production observed was 72 and 50 ml/g Total Volatile Solids (TVS) for the DRT 07K6 *bmr*3 derivative and *bmr* 3 (IS 21888) respectively. Acetate and butyrate were accounted >85% of volatile fatty acids, indicating acid type fermentations. Statistical analysis revealed that all *bmr* mutant derivatives with respect to source differ significantly in cumulative biohydrogen production, plant height, grain yield and lignin content. Biohydrogen production from biomass associated at least two different levels, one at lignin entanglement another at the polymeric nature of cellulose and hemicellulose. Further studies are necessary to determine the effect of biomass structure associated with different *bmr* traits on the microbial growth and biohydrogen production rate.

1. Introduction

Agro-industrial biomass material is considered to be the best source of carbon for bioenergy production because they are renewable and abundantly available globally [1–5]. Biomass or crop residues consist of polymeric hemicelluloses (mainly xylans) and cellulose ranging in chain lengths of up to 70–80 and that are entangled with small amounts of lignin (12–15%) [4–6]. Biomass utilization as basic raw material for biotechnological products depends on efficiency of conversion to

* *Corresponding author*. Tel.: +91 40 27191664; fax: +91 40 27193159.
E-mail addresses: prakasam@iict.res.in, Prakasham_r@yahoo.co.in (R.S. Prakasham).
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monomeric carbohydrates and disentanglement from lignin that is recalcitrant to microbial degradation [5]. Though several pretreatment approaches have been developed, most of these are efficient in breaking polymers but also produce new chemicals due to lignin degradation and interaction of produced monomeric carbohydrates which are toxic to microbial growth [5]. These inhibitory compounds reduce productivity yields in further fermentations. An alternative approach is biodegradation by carbohydrases, i.e. the cellulases and xylanases. The major problem that cannot be overcome by enzymatic degradation is the steric hindrance where lignin limits access by cellulases. Although the lignin degrading peroxidases are known, they have high energy requirements and only work in living microorganisms in direct contact with lignocellulose, and cannot be used efficiently as process enzymes [6,7]. Hence, development of new crops and cultivars needs to target for lowering lignin content to improve biofuel production efficiency from biomass [8–10].

Among different agro-industrial biomass materials, sorghum stalks *per se* serves as an excellent feedstock for biofuel due to high daily biomass production compared to other crop sources [3–5,8,9,11–14]. The efficiency of these feedstocks for biofuel production depends on microbe ability to hydrolyze cellulose, hemicellulose and ferment to a biofuel which is restricted due to the presence of lignin [5]. Research on cultivar improvement for easily digestible sorghum produced a brown midrib (*bmr*) cultivars characterized by brown vascular tissue and have significantly lower levels of lignin content (51% less in stems and 25% less in leaves) due to spontaneous mutations in any one gene of lignin biosynthetic pathway. It was reported that a 50% higher yield of fermentable sugars from stover of certain sorghum *bmr* lines was observed after enzymatic hydrolysis (www.ct.ornl.gov/symposium/index_files/6Babstracts/6B_01.htm). The International Crops Research Institute for the Semi-arid Tropics (ICRISAT) developed *bmr* sorghum hybrid parents (involving the mutants *bmr* 1, *bmr* 3 and *bmr* 7) characterized by high biomass yields. So far these *bmr* derivatives have been evaluated for animal feed [9,12], however, they can also be exploited as substrate materials for bioenergy production as these lines have a more favorable chemical composition (low lignin levels). Little information is available on utilization of *bmr* sorghum biomass as feedstock for biohydrogen production. Based on the above considerations, the aim of the present study was to evaluate the effect of low lignin content in *bmr* mutant derivatives for biohydrogen production by anaerobic fermentation using buffalo dung compost as inocula.

2. Materials and methods

2.1. bmr *lines*

Three improved *bmr* 3 derivatives namely DRT 07K1, DRT 07K6 and DRT 07K15 in different agronomic backgrounds were developed following recurrent backcrossing method at ICRISAT and the same were used along with the parental source mutant *bmr* 3 (IS 21888) in this study. Estimation of metabolizable energy of biomass was performed according to [9]. Lignin and other components of biomass were measured as per the established protocols [10,11].

2.2. *Natural inoculum and pretreatment*

H_2-producing mixed consortia inoculum was developed according to Ref. [5] briefly, hydrogen-producing mixed consortia that originated from buffalo dung compost was collected in Hyderabad city, Andhra Pradesh, India. To deactivate the hydrogenotrophic methanogens and to enrich the hydrogen-producing spore-forming anaerobes, the buffalo dung compost was subjected to heat treatment for 30 min at 100 °C. This inoculum was stored under anaerobic environment for further use.

2.3. *Experimental procedure*

Fermentation experiments were performed according to Prakasham et al. [5], in 250 ml serum vials as batch reactors consisting of pre-treated buffalo compost slurry (15%) – 15 ml, 5 g *bmr* sorghum stover material, 15 ml of nutrient stock solution (prepared using the following composition (in g/L) NH_4Cl – 0.5, KH_2PO4 – 0.25, K_2HPO_4 – 0.25, $MgCl_2.6H_2O$ – 0.3, $FeCl_3$ – 0.025, $NiSO_4$ – 0.016, $CoCl_2$ – 0.025, $ZnCl_2$ – 0.0115, $CuCl_2$ – 0.0105, $CaCl_2$ – 0.005 and $MnCl_2$ – 0.015). The final working volume of 150 ml was made up with distilled water. These flasks were deoxygenated with nitrogen gas for the development of an anaerobic environment. These flasks were incubated at 37 ± 1 °C in an orbital shaker with a rotation speed of 100 rpm to provide better mixing of the substrates. The volume of biogas produced was determined using glass syringes of 5–50 ml. All the experiments were performed in triplicates and the average values were reported.

2.4. *Chemical analysis*

The hydrogen gas measured as a percentage of the total volume was determined using a 100% hydrogen standard with gas chromatograph (GC, Agilent 4890D) equipped with a thermal conductivity detector (TCD) and 6 feet stainless column packed with Porapak Q (80/100 mesh). The operational temperatures of the injection port, the oven and the detector were 100 °C, 80 °C and 150 °C, respectively. Nitrogen gas at a flow rate of 20 ml/min was used as the carrier. VFA estimation was performed according to Ref. [3].

3. Results and discussion

3.1. bmr *derivatives and their characterization*

Three low lignin containing *bmr* 3 derivatives namely DRT 07K1, DRT 07K6 and DRT 07K15 along with *bmr* 3 (IS 21888) were evaluated for their suitability to biohydrogen production by anaerobic fermentation. These three *bmr* allele introgressed derivatives were selected based on their better agronomic performance compared with the parent, *bmr* 3 (IS 21888) in terms of grain yield, plant height and lignin content (Table 1). The improved *bmr* 3 derivatives were characterized with higher plant height (>1.9 m), grain yield (>1.4 t/ha) and also

Table 1 – Properties of developed *bmr* lines and fermentative biohydrogen production along with statistical significances.

S. No.	Entry	Cumulative biohydrogen (ml/g TVS)	Plant height (m)	Grain yield (t/ha)	Metabolizable energy (kcal/kg)	Lignin%	Total biogas (ml/g TVS)
1	DRT 07K1	68.04 ± 2.07	1.9 ± 0.05	2.1 ± 0.06	7.07 ± 0.21	3.07 ± 0.09	128 ± 3.84
2	DRT 07K6	72.0 ± 2.98	2.0 ± 0.06	1.4 ± 0.03	7.38 ± 0.22	2.85 ± 0.08	135 ± 4.05
3	DRT 07K15	59.22 ± 2.10	1.9 ± 0.05	2.5 ± 0.07	7.18 ± 0.21	3.44 ± 0.10	111 ± 3.35
4	*bmr* 3 (IS 21888)	50.40 ± 1.98	1.6 ± 0.04	0.6 ± 0.01	6.79 ± 0.20	3.96 ± 0.12	96 ± 3.36
Analysis of variance for agronomic traits							
Mean Sum of Squares			222.66[b]	0.48[b]	3.74[b]	0.01	
Standard error			2.33	0.01	0.01	0.03	0.19
Correlation of agronomic traits with cumulative biohydrogen production							
Cumulative biohydrogen production			1.000				
Plant height			−0.908[b]	1.000			
Grain yield			−0.308	0.509	1.000		
Metabolizable energy			−0.178	−0.253	0.371	1.000	
Lignin			0.69	−0.755[a]	−0.715[a]	0.189	1.000

a Significant at $P < 0.05$.
b Significant at $P < 0.01$.

reveals that they differ in lignin content compared to their parent, *bmr* 3 (IS21888). The lowest lignin content was observed in DRT 07K6 (2.83%) and the highest was recorded in DRT 07K1 and DRT 07K15 (3.01% & 3.44% respectively) which was less than the *bmr* 3 (IS 21888) (3.96%) indicating that these may be more effectively degraded during fermentation due to the low lignin content [4]. In addition, all the derivatives were characterized with higher metabolizable energy values ranging from 7.07 to 7.38 kcal/kg where as the parent *bmr* 3 (IS21888) has only 6.79 kcal/kg. These were considered as important factors for effective hydrolysis by microbial strains [8,9].

3.2. *Biohydrogen production*

Fermentation experiments were performed at different pH values by varying the solid to liquid ratio at 37 °C along with a supplementation of nutrient stock solution. The maximum biogas production was observed at pH 6.0 with 1:28 solid to liquid ratio (data not shown). Fermentation conditions have been shown to influence microbial products as observed in the literature [2–4,13,15–18]. Hence, further experiments were performed using 3.5% (w/v) of dry biomass as carbon source at 37 °C for all the sorghum samples. After 36 h of incubation, the biogas was collected and analyzed for the hydrogen content.

The data indicated that all selected *bmr* lines were suitable for biohydrogen production. However, the quantity of biohydrogen produced ranged from 50 to 72 ml/g TVS. The study of Shi et al. [14], also demonstrated higher quantities of biohydrogen 52.1 ml/g TVS with raw sweet sorghum as a substrate material. This is interesting because, the selected *bmr* derivatives have significantly lower lignin content by up to 50% [11] yet the biohydrogen production presented in this study is higher (72 ml g^{-1} TVS with DRT 07K6) (Table 1) than that of sweet sorghum biomass where authors reported 52.1 ml/g TVS [14] indicating the importance of lignin content of substrate material for biohydrogen production. Prakasham et al. [13], and Mangnusson et al. [11], are also concluded that the variation in production is dependent on the substrate or feedstock type and other fermentation factors.

The maximum (72 ml/g TVS) and minimum (50 ml/g TVS) amount of cumulative hydrogen gas was noticed with fermentations performed using DRT 07K6 and *bmr* 3 (IS21888), respectively, followed by DRT 07K1 (68 ml/g TVS) and DRT 07K15 (59 ml/g TVS) (Table 1). This data suggested that the source *bmr* 3 (IS 21888) contains higher lignin content prevented biodegradation due to a steric hinderance to the cellulolytic enzymes produced by microbial consortia, thus reduces biohydrogen production. In addition among different *bmr* derivatives increased amount of lignin content reduces biohydrogen production under similar fermentation conditions. In fact, availability of carbon is considered as one of the major factors for effective biohydrogen production [3,5,13]. For each percent less lignin, two to four times more cellulose is available to bioreactor carbohydrases when acid/heat pretreatment are not used, or require less acid and heat to release the same amount of available carbohydrate [7]. Similar variability of hydrogen production data was noticed with other agro-industrial biomass materials [2,13,14]. Shi et al also reported higher biohydrogen (>127 and 181 ml g^{-1} TVS) with alkali treated sweet sorghum stalks and molasses based substrates, respectively where more available carbon for the biohydrogen production was expected [14]. This suggested a limitation associated with microbial consortia in hydrolysis of biomass. This is further confirmed by observed variation in total biogas production (Table 1) in the present study. The noticed differences in biohydrogen production as well as lignin content of *bmr* 3 derivatives denoted that recalcitrant nature of biomass associated at least two different levels, one at lignin entanglement another at the polymeric nature of cellulose and hemicellulose.

The volatile fatty acids (VFAs) production is always associated with H_2 fermentations [5]. During our experimentation the acetate and butyrate accounted for >85% of total VFA concentration, but propionate concentration was low (Fig. 1). These H_2 yields are in agreement with those in biohydrogen fermentation from glucose, in which VFAs mainly consists of acetate and butyrate. However, further comparisons of the dynamics of VFAs production with microbial consortia would

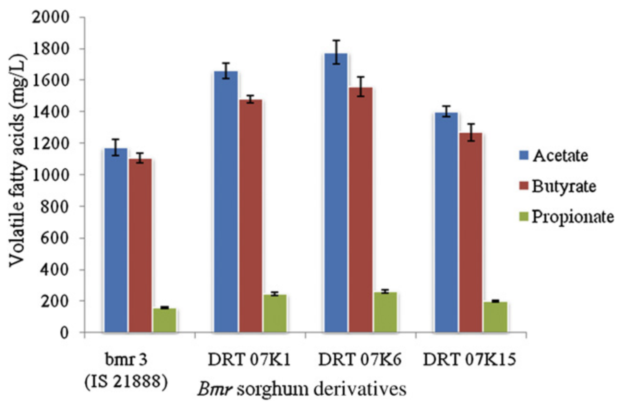

Fig. 1 – Volatile fatty acid production profile during fermentative biohydrogen production.

provide better understanding of mechanisms the biochemical reactions involved in H_2 production with different *bmr* derivatives.

The analysis of variance (Table 1) revealed that the genotypes evaluated were significantly different for all the traits under study, i.e. total biomass, cumulative biohydrogen production, plant height, grain yield, lignin content and metabolizable energy. Therefore, further analysis was performed to find out the most influential agronomic traits on biohydrogen production through correlation analysis. A perusal of data indicated that cumulative biohydrogen production is significantly and negatively influenced by lignin content (Table 1). Surprisingly there was negative correlation with plant height. Therefore, one needs to develop low lignin content biomass lines to realize higher quantities of hydrogen.

4. Conclusions

Brown midrib sorghum stalks *per se* as a novel material for anaerobic biohydrogen production was investigated using three *bmr* 3 derivatives associated with variation in lignin content and compared with source parent mutant *bmr* 3 (IS 21888) under similar fermentation environment. Biohydrogen production differed among different *bmr* genotypes which contain varying amounts of lignin content and revealed inter-relation between lignin content and biodegradation of sorghum stover materials. Genetic improvement toward low lignin content can improve the production of biohydrogen and there by reduce the cost of pretreatment for solid agrowaste materials. Further research should involve a detailed study at microbial consortia, their metabolic flux and structural variation that relates to *bmr* traits involving hemicellulose and cellulose will provide basic information which could be effective for up-scaling biohydrogen production.

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