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Production of Reducing Sugars from Hydroxylated Mature Spear Grass (*Heteropogon Contortus)*

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ABSTRACT: Mature black Spear grass (*Heteropogon contortus*) was hydrolyzed using different concentrations of dilute sulphuric acid for the first time as lignocellulosic substrate for producing reducing sugars at different pH and retention time. The maximum total reducing sugar (TRS) from Spear grass hydrolysate (SGH) was 2.48g/L using 6% H_2SO_4 at 121ºC for 4h with higher amount of xylose than glucose and arabinose. BC yield was comparable to those from reported lignocellulosic hydrolysates. The result of the study proved that mature back spear grass, commonly known as tanglehead, which is less useful as fooder due to lower nutrient, clogs animal furs and, sticks to human walker's clothes, can be hydrolyzed with little cost to produce fermentable sugars used as substrates for producing such biotechnological products as, biofuels, biogas, and bacterial cellulose.

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Lignocelluloses are present in biomass or non-food crops and comprise hemicelluloses (hC), cellulose (C) and lignin (L). Amount of cellulose, hemicelluloses and lignin in grasses ranges from 25-40%, 35-50%, and 10-30% respectively (Malherbe and Cloete, 2002). Hemicellulose is highly branched lignocellulose with short side chains containing 6-Carbon sugars (hexoses) as well as 5-Carbon sugar sugars (pentoses) (Kumar, 2009). On the other hand, cellulose is a homopolymer consisting of repeating branched glucan units. Lignin is a heteropolymer containing sugar and other aromatic alcohol. Pretreatment of the lignocelluloses is important in order to disintegrate the matrix holding the components together and hence makes it easier for lignocellulose components to be hydrolyzed. Hydrolysis is a process of breaking down the complex polymer into their simple fermentable forms in order to make it readily available for microorganisms to metabolize. Pretreatment can be achieved by a diverse method including use of acid, alkali, steam, or microorganism or their enzymes (Kumar, 2009).

Biomass lignocelluloses contain quite a good amount of fermentable sugars (Jørgensen *et al*, 2007; Binder and Raines, 2010; Mezule *et al,* 2015). When a disaccharide is used as carbon source, they are firstly hydrolyzed to the monosaccharide. For example, sucrose (cane sugar) and maltose are hydrolyzed into glucose plus fructose and two glucose molecules respectively. Furthermore, inter-conversions of sugars are also possible because of the enzyme potentials in the microorganisms involved. For example, fructose can be isomerized to glucose; mannitol is reduced to fructose while xylose is reduced to xylitol. During hydrolysis, lignocelluloses are broken down to cellulose, hemicellulose and lignin. Cellulose (a polysaccharide) is hydrolyzed to cellobiose (oligosaccharide), which on further hydrolysis gives glucose, while lignin yields phenols (aromatic alcohols) and sugars. On the other hand, hemicelluloses yield a mixture of pentose and hexose sugars as well as uronic acid.

Acidic, Alkaline and /or enzymatic hydrolysates of many lignocellulosic biomass -plants or plant residues have been used as feedstock for the production of industrial chemicals including bioethanol and biogas as well as some organic acid (Hernández *et al,* 2012; Wang *et al*, 2013; Anwar *et al*, 2014; Scholl *et al,* 2015) and biocellulose (Chen *et al,* 2013). While bioethanol and biogas are renewable energy resources, with high premium over gasoline, biocellulose is used in both food, medical and industrial applications

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(Kiziltas *et al*, 2015; Moniri *et al*, 2017; Picheth *et al*, 2017).

Black spear grass (*Heteropogon contortus*) is a tropical perennial grass that belongs to the grass tribe, Andropogonea. It grows to a height of 1.5m. SG has green or greenish blue leaves with blade folded when young but flat when matured. The leaf is approximately 3-30 cm long and 2-8 mm wide, and somewhat canoe-shaped. SG can only be used as fodder when young and not when matured because of the lesser nutritive value of the latter and rough edges (Mohd Kassim et al, 2015). On maturity, SG becomes a health hazard as the sharp-pointed seeds and tangled awns may injure animals and humans (Cook et al, 2005; Soromessa, 2011). More so, it becomes a nuisance to humans as its seeds stick to socks and clothes of persons or animals that walk through it (Ansong and Pickering, 2013), which may take the individual ample time to remove and hence it is locally referred to as "assignment grass" although, this may be one of the ways it can be dispersed to other environment. The objective of the study was to produce reducing (fermentable) sugar from spear grass (SG) using different concentrations of dilute sulphuric acid at different retention time. SG was chosen because it is readily available in most fields and farms and requires no cost for its harvest. Also, it is rich in glycosides, flavinols, minerals and vitamins. The nutritional datasheet of SG is given by Heurze *et al* (2017).

MATERIAL AND METHOD

*Collection and Preparation of Spear grass hydrolysate***:** Mature Spear grass (SG) (Figure 1) was harvested from a farm along Omoku-Ahoada road, Omoku. Nigeria [5°19'4'N: 6°39'8'14° E]. They were cut into smaller bits and air-dried in a shady place for 5 days. Dried SG was powdered with a kitchen blender (model, Philip). SG hydrolysate was prepared according to Vasquez et al (2010) with some modifications. Briefly, 30g of grounded SG was pre-treated by steaming at 95°C for 3 hours in a water bath (GenLab Ltd, 20-100°C) and cooled to 25°C. Acid hydrolysis was done by adding 1.5L of 2.0%, 4.0% and 6.0%v/v and 8% v/vH_2SO_4 (Sigma-Aldrich, Germany) respectively to the pre-treated SG at 121 °C and different time protocols (2, 4, 6 and 8h). SG hydrolysate was allowed to cool, filtered by Whatman filter ™ paper, no 1. The filtrate was stored in the dark cupboard until it was used and the residue which is believed to contain lignin and small quantities of other indigestible compounds were discarded.

Determination of Total reducing sugar and Sugars present: Types of reducing sugars present in Spear grass acid hydrolysate was determined by thin layer chromatography (TLC) using n-butanol: acetic acid: water (4:1:5) as running solvent followed by spraying of dried plate first with silver nitrate (0.1ml) solution an acetone, and then with 0.5N sodium hydroxide resorcinol-butanol. Briefly, RB reagent was prepared by dissolving 1.0g of resorcinol in 50ml of n-butanol and 0.25N hydrochloric acid.

Fig 1. Black Spear grass (Heteropogon contortus)

A quantitative test was carried out to determine the concentration of the reducing sugars and hence effect of acid concentration and reaction time on the fermentable sugar obtained by the hydrolytic process. Reducing sugar was determined by the 3.5-dinitrosalicylic acid (DNS) method of Miller (1956) as described by Guskavo et al, 2011) with slight modification. Reducing sugar is identified by the presence of the carbonyl $(-C^{=O}_{-H})$ and keto group $(-C-C^{=O})$ in aldehyde and ketone respectively. Briefly, 3ml of DNS reagent (Lab M, India) was added to 3.0ml SGH in 250ml conical flask and heated over water bath at 90°C for 19 minutes for the development of reddish brown colour. Then, 1.0 ml of Rochelle salt (potassium sodium tartarate) solution prepared by dissolving 4.0g in 100ml of de-ionized water was added to the flask, cooled to room temperature and the optical density read at 540 nm with a Colorimeter (Model 5051, Jenway Ltd) after calibration with standard solutions of the sugars detected using different concentrations of 0.1.- 1.0%

*Statistical Analysis***:** Data obtained were subjected to two-way analysis of variance (ANOVA), Pearson Correlation analysis was carried out to determine how the amount of total reducing sugar in SGH varied with the concentration of the sulphuric acid used and time of hydrolytic reaction. All analyses were conducted by MS Excel data analysis tool Pak and SPSS, version 18. Conversion of units was done using online converter software.

RESULT AND DISCUSSION

Amount of Lignocelluloses and Reducing Sugar in Spear grass Hydrolysate: Amount of lignocelluloses in SGH (%), total reducing sugar (TRS) and individual sugars are given in Figures 2 and 4 respectively. The SGH used for his study contained the following lignocelluloses (%): 22.86 hC, 15.98 lignin and 39.96 Cellulose, thus there was more cellulose than hemicelluloses and lignin (Figure 2). This result is similar to those reported of another species of spear grass (cogon grass) which had 27.13, 5.67 and 37.13% hC, L and C respectively (Kassim et al, 2015), In a related study, elephant grass hydrolysate (EGH) used for BC production was reported to have lower amount of hC (22.63%) and lignin (10.56%) but higher percentage of cellulose (43.03%) (Yang et al, 2013) when compared to SGH. Other results of lignocelluloses from acid hydrolysates or acid and enzymatic hydrolysates include Switch grass (*Panicum virgatum*)- 45% (hC), 31.4% (C) and 12 % (L); Bermuda grass-25% (hC), 36% (C) and 6.4% (L) (Jørgensen et al, 2007); *Kikuyu* grass (*Pennisetum clandestinum*)-26.2% (hC), 26.9% (C),5.86% (L) (Cardona and Rios, 2012) and Lemon grass (*Cympobogon citratus*)- 28.5-47% (hC), 29.9-35% (C),7-11% lignin (L) (Hussin et al, 2016).

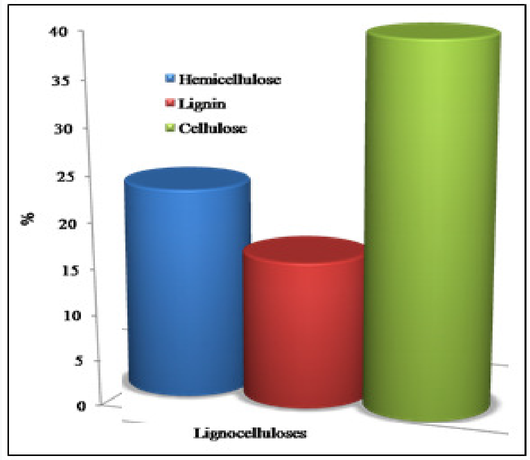

Fig 2. Percentage of lignocelluloses in spear grass hydrolysate

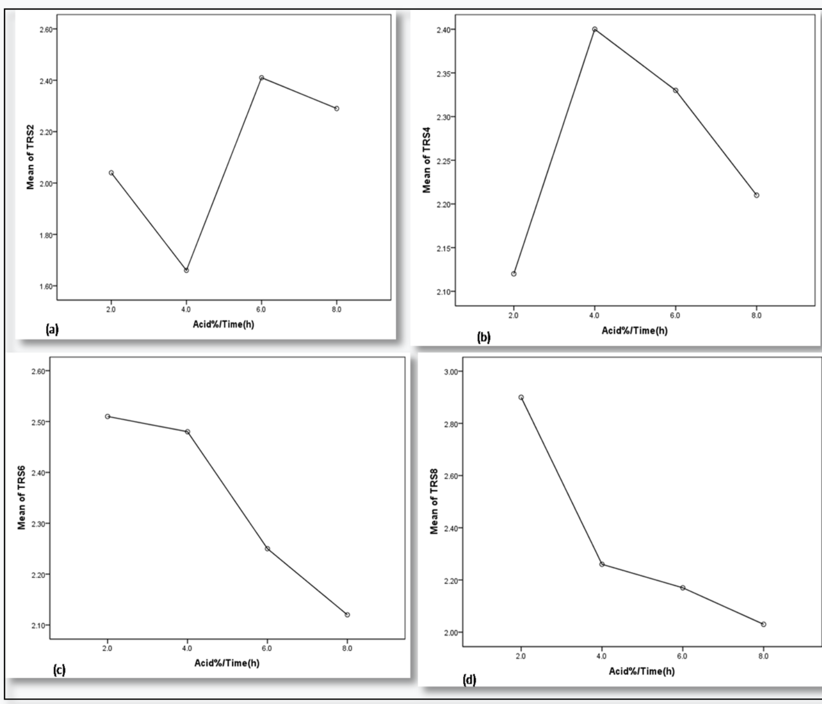

Fig 3(a-d). Effect of H_2SO_4 Concentration (%) and Reaction Time (h) on Mean Total Reducing Sugar (TRS) (g/L) in Spear Grass Hydrolysate as determined by DNS Method (a) 2%acid (b) 4%acid (c) 6% acid (d) 8%)

Table 1. Two –way ANOVA on the mean Total Reducing Sugars produced following acid hydrolysis

Source of Variation	SS	df	MS	F	P-value	F crit
TRS	0.00045	1	0.00045	0.022632	0.889964	10.12796
Acid conc/Time	0.01645	3	0.005483	0.275775	0.840915	9.276628
Error	0.05965	3	0.019883			
Total	0.07655	7				

Table 2. Correlation Analysis of Total Reducing Sugar (TRS) in Spear Grass Hydrolysate, Acid Concentration and Reaction Time

		TRS2	TRS4	TRS6	TRS8	Acid%/Time(h)
TRS(2%)	Pearson Correlation	1	-.331	-.761	-.287	.584
	Sig. (2-tailed)		.669	.239	.713	.416
	N	4	4	4	4	4
TRS(4%)	Pearson Correlation	-.331	1	.007	-.598	.207
	Sig. (2-tailed)	.669		.993	.402	.793
	N	4	4	4	4	4
TRS(6%)	Pearson Correlation	-.761	.007	1	.775	-.966*
	Sig. (2-tailed)	.239	.993		.225	.034
	N	4	4	4	4	4
TRS(8%)	Pearson Correlation	-.287	-.598	.775	1	-.905
	Sig. (2-tailed)	.713	.402	.225		.095
	N	4	4	4	4	4
	Pearson Correlation	.584	.207	-.966*	-.905	1
	Sig. (2-tailed)	.416	.793	.034	.095	
	N	4	4	4	4	4

Effect of Sulphuric acid concentration and Retention time on Total reducing sugar: Acid hydrolysis of pre-treated black spear grass using 2% sulphuric acid (H_2SO_4) yielded 2.04g/L, 1.66g/L, 2.41g/L and 2.29g/L TRS at 2h, 4h, 6h and 8h respectively (Figure 3). In general, TRS seems to reduce as the retention time increased, although hydrolysis maintained for 2hours gave a proportionate increase from 2.04 to 2.90g/L but dropped to 2.26g/L in 4h. TRS at 4% acid increased from 2.12 g/L in 2h to 2.40g/L in 4h and then decreased to 2.21 in 8h. The best acid concentration and time regime that gave the highest reducing sugar (2.48g/L) was 6% at 4h (Figure 5). The value obtained is although lower than 2.53g/L of TRS with 6% dilute H_2SO_4 after 6h hydrolysis reported by Vasquez et al (2010) for *Kikuyu* grass, but higher than 0.51g/g reported with 3.0% H_2SO_4 at 1.5h pretreatment and enzyme hydrolysis (Nigam, 2002) and 0.54g/g TRS in Ans grass *(Saccharum spontaneum)* with a total carbohydrate of 64.7% (Singh et al, 2011). F statistic indicates that there was no significant difference in the TRS obtained at the different H_2SO_4 concentrations and time allowed for the hydrolysis of SGH, $F(1, 7) = 0.023$, $p>0.05$ (Table 1).On the contrary, Idrees et al (2014) reported a significant difference on the effect of acid concentration on TRS. Correlation analysis shown in Table 2 indicates only a significant negative correlation of TRS at acid concentration 6% and 6h , $r(4) = -0.966$, $p\ 0.034<0.05$). The types of sugars detected by TLC included arabinose, glucose and xylose as shown in Figure 4 DNS assay showed higher amount of xylose (21.2 ± 9.02) than glucose (15.2 ± 4.7) and lesser amount of arabinose (2.27 ± 0.07) g/L. The maximum TRS in SGH was 2.48g/L (55.9%). TRS content in hydrolysates varies according to the type of pre-treatment and hydrolytic agent employed as given by Binder and Raines (2010). Pre-treatment is meant to release the hemicelluloses and cellulose from the lignin backbone and render it available for acid hydrolysis in order to produce the oligo –and monosaccharides used for bioconversions.

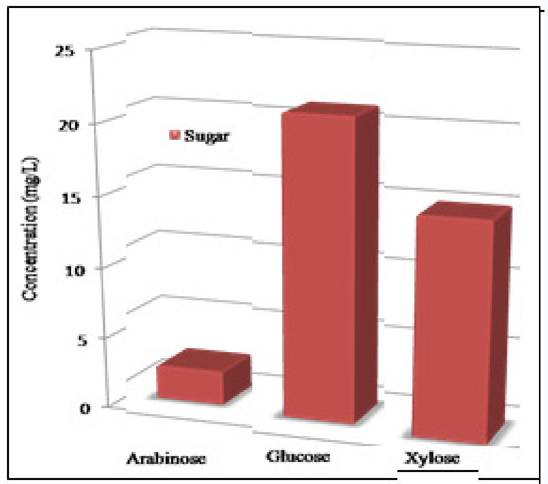

Fig 4. Types and amount of sugars in spear grass hydrolysate used for Biochemical production

The individual sugars obtained in this study though higher were similar to the trends in EGH, which had 2.31g/L arabinose, 12.0 g/L glucose and 20.3g/L xylose (Yang et al, 2013). Switch grass hydrolysate also had higher glucose (30.2g/L) but lower xylose content (10.5g/L) respectively (Sun et al, 2013). On the contrary, an acid pre-treatment before enzymatic hydrolysis of the water hyacinth, with 3.0% sulphuric acid at 121ºC and 1h retention time gave a TRS yield of 33.0g/L per 100g biomass which was used for bioethanol production (Idrees et al, 2014).

Conclusion: The study has demonstrated the potential of mature black spear grass hydrolysate as a no-cost medium for producing a reducing sugar which serve as feedstock for the production of many biochemicals. Result shows that retention time and concentration of sulphuric acid significantly affected production of RS in the SGH. The use of mature black spear grass in this way has both economic, environmental and health importance.

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