

## Original Research Article

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## Bacterial Cellulose Production from Mature Black Spear Grass Hydrolysate by *Gluconacetobacter xylinus*: Effect of pH, Fermentation Time and Nitrogen Supplementation

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### ABSTRACT

Mature black spear grass (*Heteropogon contortus*) hydrolysate (SGH) was used for the first time as lignocellulosic substrate for production of bacterial nanocellulose (BC) by *Gluconacetobacter xylinum* at 30°C and pH 5.5. The maximum total reducing sugar (TRS) from SGH was 2.48g/L using 6% H<sub>2</sub>SO<sub>4</sub> at 121°C for 4h with higher amount of xylose than glucose and arabinose. BC yield was comparable to those from reported lignocellulosic hydrolysates. At the end of 15 days, mean BC yield (g/L) was 3.84±2.23. pH produced a significant effect on BC yield (p<0.05). There was a steady increase from an initial pH4 which later declined with further increase in pH. The maximum BC yield (g/L) (4.88±1.25) occurred at pH 6. The optimal pH was 5-6. Significant effect of fermentation time (FT) and BC on one hand and pH on BC on the other hand was determined statistically using regression model. Supplementation of SGH with nitrogen sources- yeast extract (YE), peptone or both did not increase BC yield above the value observed for SGH. There was however significant difference in BC yield from basal medium supplemented with the nitrogen sources, F (3, 4) =772, p<0.05.

#### Keywords

Bacterial nanocellulose,  
Spear grass hydrolysate,  
pH, *Gluconacetobacter*  
*xylinus*

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### Introduction

Bacterial cellulose or Biocellulose (BC) or bacterial nanocellulose (BNC) is natural, pure cellulose quite different in morphology from plant cellulose.

The cellulosic pellicle formed at the surface of culture medium during the growth of the producing bacteria in a static medium is fibrillar in nature of nano dimension and hence the name, bacterial nanocellulose fibril (BNCF) to further differentiate it from the

nanocrystal form produced in agitated cultures. BC, whether nanofibre or nanocrystal is a much desired biotech material with wide applications in the paper and textile industry and highly demanded in biomedical outfits for treatment of high degree burns, artificial artery for drug delivery, scaffold for tissue engineering as well as food additives and other industrial applications (Klemm *et al.*, 2001; Chalwa *et al.*, 2009; Dahman *et al.*, 2010; Lee *et al.*, 2013; Shah *et al.*, 2013; Keshk, 2014; Zhijun *et al.*, 2014; Tsuoko *et al.*, 2015; Sulaeva *et al.*, 2015; Cacicedo *et al.*,

2016; Liyaskira *et al.*, 2017; Moniri *et al.*, 2017; Picheth *et al.*, 2017).

The standard medium for Biocellulose production, popularly known as Hestrin-Schramn(HS) medium in honour of the first developers in 1954 contains (w/v), 5% glucose, 1.6% bacto-peptone, 0.2% citric acid, 0.2% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, and 0.03% MgSO<sub>4</sub> (Hestrin and Schramn, 1954). It is incubated aerobically under static condition. Unfortunately, the high cost of the HS medium components *vis-à-vis* under-production had necessitated the search, research and adoption of earth's abundant renewable agro resources such as lignocellulosic materials, with or without supplementation for optimal production of BC.

Acidic, Alkaline and /or enzymatic hydrolysates of many 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 and even enzymes (Hernández *et al.*, 2012; Krogelli *et al.*, 2013; Wang *et al.*, 2013; Scholl *et al.*, 2015). Hydrolysates of grasses such as elephant grass, and cereal grasses such as corn, rice, wheat and barley as well as specific sugar broths have been employed as medium for BC production, with and without supplementation of the standard HS medium, with varying results as shown in Table 1. In other times, the residues obtained after the processing of agro-industrial lignocellulosic materials have also been used. There has however been no mention of spear grass or its residue hydrolysate being used as substrate in BC production except for producing fermentable sugar for fuel ethanol (Anwar *et al.*, 2014).

Black spear grass (*Heteropogon contortus*) is a tropical perennial grass that 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) (Fig. 1). In this study, SG which was previously produced by heat pretreatment followed by acid hydrolysis (Dirisu *et al.*, 2018) was used for the first time as culture medium for BC production by the bacterium, *Gluconacetobacter xylinus*, taking into accounts the kinetics for optimum yield. 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)

## Materials and Methods

### Collection and preparation of spear grass hydrolysate

Spear grass acid hydrolysate used for Biocellulose production was prepared by Dirisu *et al.*, (2018) and contained 22.86% hemicellulose, 15.98% lignin and 39.96% cellulose. Reducing sugars detected by thin layer chromatography included arabinose (2.27+0.07) g/L, glucose (15.2 +4.7) g/L and xylose (21.2 + 9.02) g/L.

### Inoculation of SGH medium

*Gluconacetobacter xylinum* (GX) previously isolated from rotten banana juice (Dirisu *et al.*, 2017) was used as the bacterial inoculums for BC production. The pH of SGH medium containing 2.48g/L TRS was adjusted to pH 5 and 8 using 2N hydrochloric acid (HCl) and sodium hydroxide (NaOH) for acidic and alkaline pH respectively. Flasks were sterilized by autoclaving at 121°C for 15 minutes g allowed to cool to 35°C and inoculated in duplicates with 0.1µl of bacterial suspension of GX. Flasks were incubated aerobically at 30°C for 15 days to observe for formation of cellulosic pellicle on the surface.

pH of the BC medium was measured at the end of the fermentation. BC yield was compared with the reported yield from other agro-allied hydrolysates.

### **Effect of supplementation of SGH with nitrogen source on biocellulose production**

SGH prepared as described above (Figure 3b) was supplemented with sterile 5.0% Yeast extract (YE), 5.0% Peptone and a combination of YE and Peptone, source of organic nitrogen and adjusted to pH 5.5. Supplemented SGH media were inoculated with 0.1ml bacterial suspension and incubated at 30°C in a static condition to observe for cellulosic pellicle, for 9 days.

### **Determination of biocellulose yield**

Biocellulose was extracted for the BC medium by alkaline method as previously described by Wu *et al.*, (2014) and Dirisu *et al.*, (2017). Extracted pellicles were dried and weighed. Mass of dry BC pellicle was expressed in g/L

### **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.

Regression model was adopted to determine the relationship between BC yield and pH on one hand and fermentation time on another. Also, effect of supplementation of SGH with yeast extract on BC production was also determined by ANOVA statistics. All analyses were conducted by MS Excel data analysis tool Pak and SPSS, version 18. Conversion of units was done using online converter software.

## **Results and Discussion**

### **Biocellulose yield in Spear Grass Hydrolysate (SGH) medium at different Fermentation Time**

Biocellulose yield (g/L) ( $\bar{x} \pm \delta$ ) at the end of 15 days was 3.84±2.23 (Figure 2). BC yield increased with time of incubation and ranged from 2.8±0.14 to 5.95±0.07. Between group ANOVA on mean BC yield with time was not significant, F (1, 10) =0.037, p0.85>0.05, (Table 1). The BC yield obtained in this study is lower than 6.4 g/L in elephant grass hydrolysate at the end of 14days fermentation in a static condition (Yang *et al.*, 2013), and 5.0g/L in casein hydrolysate (Ramana *et al.*, 2000), very close to 4.0g/L in corn cob hydrolysate (Huang *et al.*, 2015) and higher than olive mill hydrolysate (0.85-2.5g/L) and Bagasse hydrolysate (1.09 g/L) (Table 3).

The effect of fermentation time (FT) of BC production as obtained from Table 1 is given by the regression equation (1):

$$BC' = 0.278 FT + 2.105 \dots \dots \dots (1)$$

The linear relationship between FT and biocellulose yield (BC') in spear grass hydrolysate was significant, F(1,3)=73.46, p 0.003<0.05 (Table 2). The 95% confidence interval for the slope, 0.175 to 0.382 also indicates that fermentation time is significantly positively related to BC'. A correlation of r = 0.98 suggests a strong negative correlation and t statistics for regression was not significant, t ((4) =6.51, p0.007>0.05).

### **pH and pH changes accompanying BC production in spear grass hydrolysate medium with time**

Changes in pH accompanying Biocellulose production in SGH is shown in Figure 3.

**Table.1** One –way Analysis of Variance (ANOVA) on bacterial nanocellulose fibril yield (g/L) with fermentation time in spear grass hydrolysate Medium

ANOVA						
Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	0.19	1	0.1875	0.037484	0.850359	4.964603
Within Groups	50	10	5.0021667			
Total	50.2	11				

**Table.2** Linear regression of fermentation time effect on BC yield (BC') in Spear grass hydrolysate medium

	Coefficients				95% Confidence Interval for B	
	B	Standard Error	t Stat	P-value	Lower	Upper bund
Intercept	2.105	0.323	6.514	0.007	1.077	3.133
Fermentation Time (FT)	0.278	0.032	8.571	0.003	0.175	0.382

**Table.3** Biocellulose yield in spear grass hydrolysate compared with different agro residues and hydrolysates

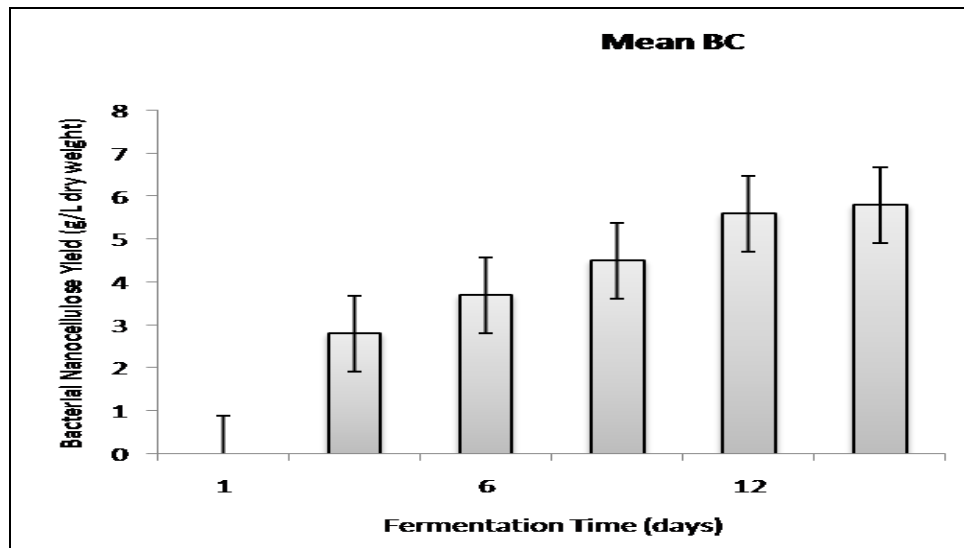
Medium	Bacterial Producer	BC yield (g/L)	Reference
Caesin hydrolysate	<i>Acetobacter xylinum</i>	5.0	Ramana (2000)
Konjac powder hydrolysate	<i>G. xylinum</i> ATCC23770	2.1	Hong and Qui (2008)
Pineapple peel juice	<i>Ga. Swingsii</i>	2.8	Castro <i>et al.</i> , (2011)
HS medium + TS	<i>Ga. Xylinus</i>	10.38	Wu and Liu (2012)
Wheat straw hydrolysate	<i>Ga. Xylinus</i>	6.3	Chen <i>et al.</i> , (2013)
	<i>Ga xylinus</i>	9.7	Al-Abdallah and Dahman (2013)
Olive mill residue hydrolysate	<i>G. saccari</i>	0.85	Gomes <i>et al.</i> , (2013)
Thin stillage (TS)	<i>Ga. Xylinus</i>	6.26	Wu (2013)
Elephant grass hydrolysate	<i>G. xylinus</i> CG001	6.4	Yang <i>et al.</i> , (2013)
Glycerol from grape bagasse	<i>G. xylinum</i>	8.0	Vasquez <i>et al.</i> , (2013)
Waste beer yeast hydrolysate	<i>G. hansenii</i> CGMCC3917	7.02	Lin <i>et al.</i> , (2014)
Corn cob hydrolysate	<i>Gluconacetobacter xylinus</i>	4.0	Huang <i>et al.</i> , (2015)
Pulp mill hydrolysate	<i>G. xylinus</i>	0.15	Kiziltas <i>et al.</i> , 2015
Sunflower meal hydrolysate + glycerol	<i>Komagateibacter sucrofermentarius</i>	13.3	Tsuoko <i>et al.</i> , (2015)
Molasses hydrolysate	<i>G. intermedius</i>	12.6	Tyagi and Suresh (2016)
Pineapple fruit juice	<i>Acetobactr pasteutianus</i>	0.38	Adebayo-Tayo <i>et al.</i> , (2017)
Baggase hydrolysate	<i>Ga. Xylinus</i>	1.09	Qi <i>et al.</i> , (2017)
Bagasse Acetic acid hydrolysate	<i>A. xylinum</i>	2.13	Cheng <i>et al.</i> , (2017)
Spear grass hydrolysate	<i>G. xylinus</i>	4.88	This study

\*Compiled from various sources

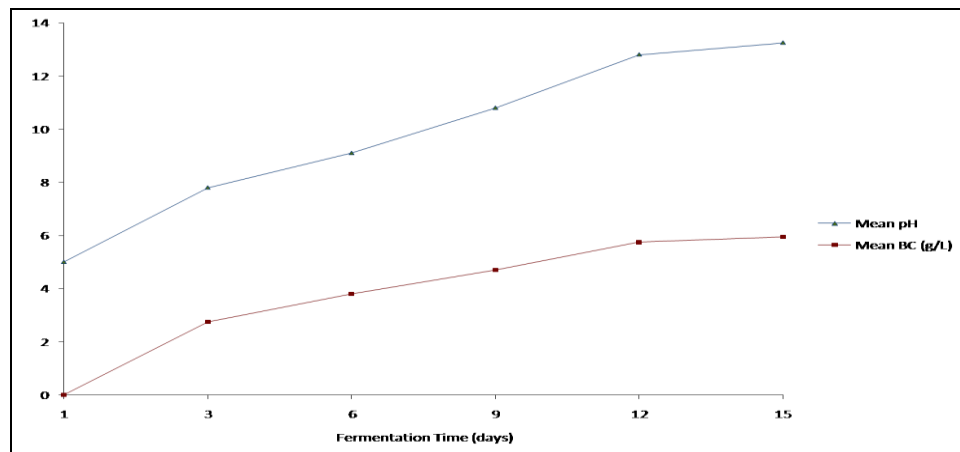
**Fig.1** Black Spear grass (*Heteropogon contortus*)



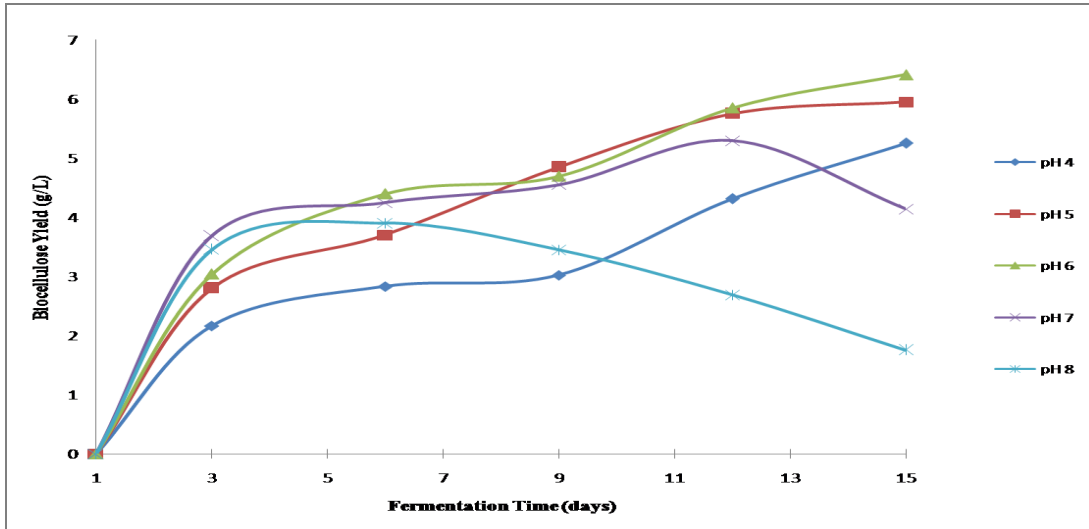
**Fig.2** Mean Biocellulose yield by *Gluconacetobacter xylinus* in spear grass hydrolysate Medium



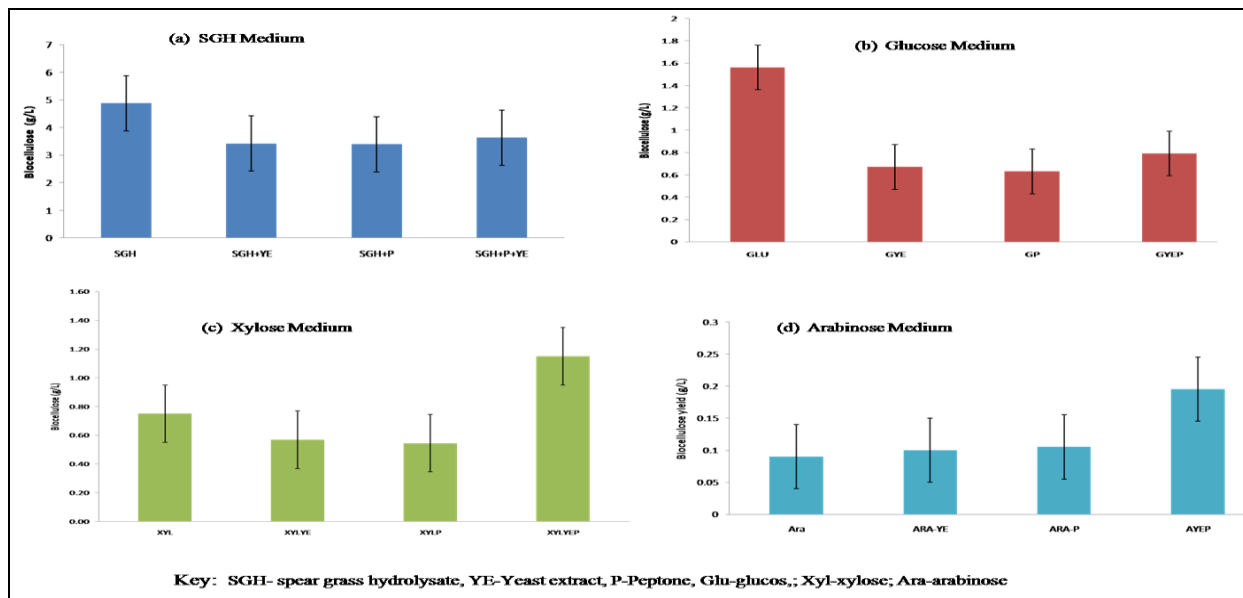
**Fig.3** Biocellulose production and pH changes in Spear grass hydrolysate medium



**Fig.4** Effect of pH on biocellulose yield in spear grass hydrolysate medium



**Fig.5** Mean biocellulose production in black spear grass hydrolysate and sugar media supplemented with nitrogen sources-Yeast extract and peptone



While BC yield increased with time, pH of SGH medium equally increased from 5.0 + 0.0, day 1 to 7.3+0.14 at day 15. This trend is similar to the report by using elephant grass hydrolysate (Yang *et al.*, 2013). In contrast, Raghunathan (2013) observed no increase in the final pH of the BC medium when *G. xylinus* was cultured on medium containing D-arabinose, or D-glucose.

**Effect of pH on biocellulose production in spear grass hydrolysate medium**

Result from Figure 4 indicates that BC production increased with increasing pH (4-67 and time of incubation in SGH medium. Mean BC (g/L) produced at different pH are 3.52+1.17, 4.61+1.28, 4.88+1.25, 4.38+0.63 and 3.05+0.82 at pH 4,5,6,7 and 8

respectively. Thus neutral to alkaline pH did not favour much BC yield. This can be attributed to the fact that the bacteria thrive well in acidic medium and as such any deviation would affect if not hinder their growth. The linear relationship between pH and BC production in SGH is given by the regression equations (1) as derived from Table 1. Maximum BC yield ( $4.88+1.25$ ) occurred at pH 6 after which it declined with further increase in pH. The value obtained was higher than the maximum yield (0.15) at pH 8 by *G. xylinus* in pulp mill hydrolysate (Kiziltas *et al.*, 2015) and 2.1g/L at pH6 in HS medium as reported by Rangaswamy *et al.*, (2015).

### **Effect of nitrogen supplementation on bc production**

Grass hydrolysate contains low percentage of nitrogen with high percentage of carbohydrates. Thus, nitrogen source- yeast extract (YE) and peptone was added to SGH medium to provide amino acids and vitamins for bacterial growth. In this study, there was significant difference in the BC yield from basal medium supplemented with nitrogen sources,  $F(3, 4) = 772, p < 0.05$ . BC yield in SGH was 4.88 g/L (Figure 5). Addition of YE, Peptone or both did not increase BC yield above the value observed for SGH. Mean BC produced were 3.42, 3.38 and 3.63g/L respectively. BC yield in YE and peptone supplemented medium in this study was higher than 3.23/ 3.22g/L and 1.29/1.04g/L in Glucose-Ethanol acetic acid medium respectively (Abdelhady *et al.*, 2015), but lower than 7.0g/L and 9.0g/L reported by adding YE and peptone respectively (Pourramezan *et al.*, 2009). In a related study involving bioethanol production, Scholl *et al.*, (2015) indicated that there was no significant difference in the various nitrogen sources added to elephant grass hydrolysate. Glucose medium supplemented with a combination of

YE and peptone gave a higher yield of 0.79g/L BC followed by YE or peptone. There was however no significant difference in BC yield,  $F(2, 3) = 0.204, p = 0.83 > 0.05$ . Rangaswamy *et al.*, (2015) and Ramana *et al.*, (2000) reported maximum BC yield of 2.15g/L and 4.8g/L following 0.5% w/v supplementation of 0.5% v/v peptone respectively, while Son *et al.*, (2003) reported a BC yield with YE (2.87g/L) followed by corn steep liquor (2.59g/L) and polypeptone (2.05g/L). Combined supplementation of fructose medium with YE and peptone gave a BC yield of 4.5g/L by *A. xylinum*. Other organic nitrogen sources used for BC production media include corn steep liquor (4.63g/L) (Costa *et al.*, 2017), casein hydrolysate (4.07 and 5.0g/L) (Ramana *et al.*, 2000) and beef extract (0.25g/L) (Abdelhady *et al.*, 2015). According to Mohammedkazeem *et al.*, (2015), YE and peptone deficiencies slow down the fermentation process because it affects cell growth although it is not required for BC production. It does appear that the type of carbon source employed in BC medium with nitrogen supplementation varies. Embuscado *et al.*, (1994) supplemented fructose media with YE and peptone and obtained 7.38g/L BC but Abdelhady *et al.*, (2015) supplemented glucose-ethanol medium with YE and peptone, which enhanced BC yield (4.59g/L). In a recent study, Biyik and Coban (2017) supplemented HS medium containing glucose with YE and obtained 0.04g/L of BC. YE is said to be needed for cell growth (Kim *et al.*, 2006) and hence explains the stimulatory role in BC production. The genetics of nitrogen deficiency has been extensively studied elsewhere and appear to be linked to cellulose biosynthesis regulation through c-amp (Ross *et al.*, 1991; Chalwa *et al.*, 2009).

The study has demonstrated the potential of mature black spear grass hydrolysate as a no-

cost medium for producing a highly valued biopolymer, biocellulose (BC) by *Gluconacetobacter xylinus*, formerly *Acetobacter xylinum* in a static incubation. SGH contains hydrolysable sugars which were metabolized and converted directly or indirectly to biocellulose. Result shows that fermentation time and pH significantly affected BC production in the SGH. Supplementation with organic nitrogen sources such as yeast extract or peptone played a stimulatory role by enhancing BC production. The optimum conditions were pH 5-6. The use of mature black spear grass in this way has both economic, environmental and health importance.

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