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pH Effect and pH Changes during Biocellulose Production by *Gluconacetobacter xylinus* in *Moringa oleifera Tea*-Sugar Medium

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Authors' contributions

This work was carried out in collaboration between all authors. Author CGD designed the study, performed the experiment, performed statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors JR and EL proof read the manuscript. Author AAO managed prepared the Moringa leaves used for the research. All authors read and approved the final manuscript.

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ABSTRACT

The study was carried out to determine the effect of pH and pH changes accompanying Biocellulose (BC) production by *Gluconacetobacter xylinus* (GX) in a *Moringa oleifera* tea-sugar medium. The authors adopted experimental and comparative design for the study. *Moringa oleifera* leaves were harvested at the front of Physics laboratory, Federal College of Education (Technical) Omoku. Nigeria. Banana was purchased from vendors in Omoku market and kept for 5-7 days to allow the development of acetic acid bacteria. *Gluconacetobacter xylinus* (GX) was isolated from spoiled banana and used to inoculate *Moringa oleifera* tea-yeast extract medium to which different concentrations of sugars-fructose, glucose and a combination of glucose and fructose were added. The pH of the medium was adjusted to pH ranging from 4-8, sterilized by autoclaving at 121°C for 15min. Media were inoculated in duplicates with 0.1 ml of GX and incubated at 30°C for 15 days to observe for cellulosic pellicle. The pellicles were extracted by alkaline hydrolysis, washed and

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weighed. Changes in pH were also monitored in the course of the fermentation. The Mean BC yield were 0.175±0.12, 0.182±0.12, 0.085 ±0.05, 0.025 ±0.03 and 0.018 ±0.03 at pH 4,5,6,7 and 8 respectively. pH changes during fermentation tended towards neutral or near-neutral range while it increased from an initial value of 4 and 5 to around 6. pH 7-8 of the fermenting mixture decreased with increasing sugar concentration but in the order, Glucose-fructose medium<Glucose medium<Fructose medium. The result of this study showed that Biocellulose, which is a biopolymer used for various industrial applications can be produced from a readily and sustainable source, *Moringa olefera* leaves with control of pH and concentration of added sugar.

Keywords: Biocellulose; Gluconacetobacter xylinus; Moringa oleifera tea; pH; sugar.

1. INTRODUCTION

Biocellulose (BC) is a natural polymer originally produced by members of the Proteobacterium, otherwise referred to as Acetic Acid Bacteria and particularly, *Acetobacter, Gluconobacter*. Currently, different genera of bacteria are known to secrete extracellular cellulose, known as microbial cellulose or Biocellulose [1-5]. Additionally, AAB metabolize sugars and intermediates oxidatively to produce a variety of organic acids, notable of which is acetic acid, gluconic acid etc, which lower the pH of the medium. In order words, the bacteria are acidophilic and/or acid tolerant hence pH values outside acidic range (>6) may either inhibit or suppress the growth and activity of the bacteria [6]. The amount of Biocellulose produced may also depend on the time of fermentation of sugar employed as carbon source for its polymerization. Biocellulose is a homopolymer of repeating glucose units linked by β, 1→4 glycosidic bonds [1] as shown in Fig. 1.

BC is a novel biotechnology polymer which has wide applications such as paper and textile making, skin repair and wound dressing, scaffold material for tissue engineering, cellulose composites for reinforcement as well as for food, paper and textile industries [7-9]. Optimization for BC production therefore requires careful choice of carbon substrates and manipulation of fermentation kinetics such as pH, carbon sources, fermentation mode and time.

The carbon source is a major determinant factor in BC production because the bacteria do not metabolize all carbon sources (sugar and sugar alcohol) to cellulose due to the absence of certain enzyme(s) to hydrolyze the particular sugar or alcohol before polymerization to cellulose. For example, it was reported that the bacteria, *Acetobacter xylinum* does not ferment lactose because it lacks galactosidase [10-11] ranging from pentoses (e.g. ribose, xylose), hexose sugars (glucose, fructose) alcohols such as ethanol, mannitol and glycerol [6,12]. In addition to the type of carbon source, the concentration used in the fermentation medium as well as the time of fermentation are equally important in BC production. Biocellulose production is evidenced by the formation of a pellicle at the surface of the medium used for its cultivation. In this study, effect of fructose, glucose, and a combination of fructose and glucose was employed in Moringa tea broth-yeast extract medium at different fermentation time regimes to establish its effect on Biocellulose production by *Gluconacetobacter xylinus.*

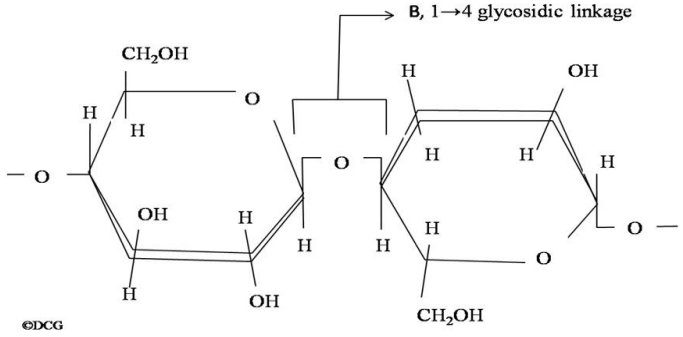

Fig. 1. Structure of cellulose monomers with glycosidic linkage between two glucose molecules

2. MATERIALS AND METHODS

2.1 Bacteria

Gluconacetobacter xylinus isolated from spoiled banana in glucose yeast extract medium containing dextrose, yeast extract in 500 ml of distilled water and adjusted to pH 7.0±0.2. with 20 µl nisin to inhibit the growth of lactic acid bacteria. Isolate was purified and characterized by microscopy, Gram stain reaction, catalase and oxidase test as well as carbohydrate fermentation [13]. The bacterium was identified as Gram negative rod with ability to form cellulosic pellicle at the surface of the medium, and have strictly oxidative metabolism. The bacterium was maintained in glucose- yeast extract broth (GYEB).

2.2 Preparation of Culture Medium

Moringa oleifera Tea broth was prepared by steeping the dried leaves in boiling water for 5minutes. It was supplemented with sterilized 5% yeast extract (Bacto) and 2.0% fructose. The pH of the medium was adjusted to give an initial pH reading of 4, 5, 6, 7 and 8 with 1N hydrochloric acid in sodium hydroxide in the acidic and alkaline pH respectively. The medium was sterilized by autoclaving at 121°C for 15 minutes, cooled to 30°C and inoculated with 20 µL of *Gluconacetobacter xylinus* suspension. Plates were incubated aerobically for 7-15 days.

2.3 Determination of pH Effect and pH Changes

Moringa oleifera tea (MOT) broth containing yeast extract was supplemented with 2.0% fructose. pH of medium was adjusted to a range of 4-8 with dilute 1N hydrochloric acid and IN sodium hydroxide for the acidic and alkaline pH respectively. pH was measured with digital pH meter (model, Jenway 3150). MOT-Fructose medium was sterilized by autoclaving at 121°C for 15 minutes, cooled and inoculated with 20 µL of bacterial suspension and incubated in static conditions at 30°C. pH was measured daily for 15 days. All tests were performed in duplicates.

2.4 Alkaline Extraction of Biocellulose

Biocellulose pellicles formed at the surface of culture medium was removed using tweezers and washed with repeated changes of distilled water. Washed pellicle were placed on filter paper to drain off and weighed to determine the wet weight of crude pellicle. The wet pellicles were transferred into fresh conical flasks and 2N NaOH solution added and left for 24 hours. Pellicles were washed with 6% (v/v) ethanoic acid until the washing reached neutral pH. Pellicles were dried in an oven at 105°C for 15 minutes. Dried pellicle were weighed and reweighed to a constant weight.

2.5 Determination of Sugar Concentration on Final pH

MOT-Yeast extract prepared as described in 2.2 was supplemented with different concentrations of sugar- fructose, glucose and a combination of glucose and fructose to a final concentration of 2%, 4%, 6% and 8% before inoculation with suspension of bacterium-*Gluconacetobacter xylinus*. The final pH of the fermenting media were determined at the end of incubation period.

2.6 Statistical Analysis

Data was analyzed with descriptive statistics. Analysis of Variance (ANOVA) was used to ascertain whether there were significant differences in mean pH values at alpha 95% level of significance. Regression analyses were performed to determine the relationship between pH and amount of BC produced with time. All analyses were carried out using Microsoft Excel Data Analysis Tool Pak (2007), Real Statistics Software and SPSS, version 18.

2.7 RESULTS AND DISCUSSION

Biocellulose production requires sources of carbon, nitrogen as well as minerals and the appropriate pH. Result of this study confirms that Biocellulose production is affected by the pH of the culture medium. Mean BC yield were 0.175±0.12, 0.182±0.12,0.085 ±0.05, 0.025 ±0.03 and 0.018 ±0.03 at pH 4,5,6,7 and 8 respectively (Fig. 2). Lower pH (4-6) favoured BC yield as there was a steady increase from 0.1 – 0.32, 0.1-0.32 and 0.09 to 0.12 from day 3-15 respectively (Fig. 3). BC production at pH 7 and 8 were minimal initially and eventually dropped to 0.0 g/l by the 15^{th} day of incubation. This is to confirm that the cellulose producing bacteria grows within acidic pH and not alkaline pH. The optimum pH therefore lies between 4-6 as also reported by [13] and [14]. BC production (0.3 g/L) was recorded at pH 4 and 5 by the end of fermentation (Fig. 3). This result is consistent

with best pH of 5 reported by [15,16] although with higher values [17] and [18] respectively; and pH 6.5, with a BC maximum yield of 0.04 and 0.029 g/L after 7days by two *Acetobacter* species [19]. Also reported of lower pH giving maximum BC yield include 4 [20,21], 4.5 [22]. pH 3 or 3.5 and below were said to inhibit bacterial growth and hence little or no BC production [23-25].

The linear relationship between pH and BC yield' is given by the BC regression equation based on least squares principles:

$$BC'(y) = a + bx \quad \ldots\ldots\ldots\ldots\ldots\ldots (1) \quad [26]$$

Where,

Y' is the mean predicted value of *Y* (BC') for a given *X (pH*.

a is the Y-intercept
b is the slope of the regression line

Regression plots obtained from Fig. 3 for linear relationship between pH and BC yield (BC') was not significant for pH 4-8 as shown in Table 1 ($p > .05$). The ANOVA were significant for BC' at pH 4-5, but not significant at pH 5-8. Coefficient of determination (R^2) values indicate that pH accounts for approximately 94%,95%, 78%, for BC production at pH 4-6, while it accounted for 4.3% and 1.3% BC yield.

3.2 Changes in pH during Biocellulose Production

Initial pH of 4 -6 gradually increased with time of fermentation while pH 7-8 led to a decrease and closing up at around 6-6.5 (Fig. 4). According to

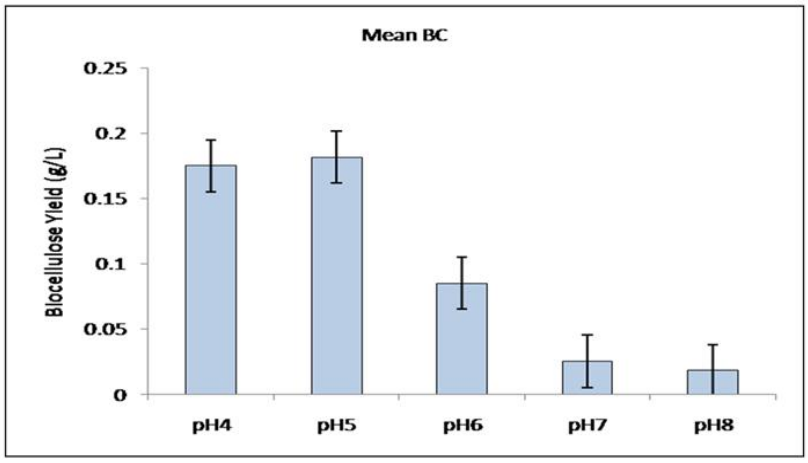

Fig. 2. Mean biocellulose yield at different pH in Moringa tea –sugar medium

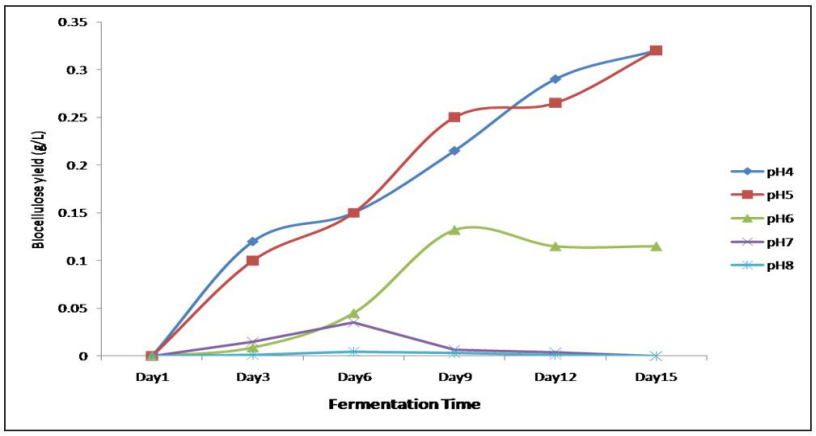

Fig. 3. Change in biocellulose yield with time of incubation at different pH in Moringa Tea –sugar medium

[24], Biocellulose production is affected by the pH of the fermenting medium. Result indicates that Biocellulose production occurred within acidic pH (4-6). The bacterium oxidized glucose to gluconic acid. Due to this pH-Biocellulose production dependence, pH of the culture medium is usually adjusted with acid or alkaline buffers at the onset of cultivation or substrates with buffering effect such as corn steep liquor [20,24] may be added to or used directly as a medium for Biocellulose production. By the end of 15days of incubation, initial pH of culture medium set at 4, 5, 6, 7 and 8 had changed to a mean pH of 4.5±0.29, 5.7±, 0.52, 6.7± 0.32, 6.5± 0.32 and 6.7± 0.63 respectively. While lower pH was rising, higher pH was decreasing and all clustering around near neutral with the exception of ph 4, which increased to near 5. This is due to production of organic acids following metabolism of the sugar.

3.3 Effect of Sugar Concentration on Final pH

Generally, there was an inverse proportional decrease of pH of the medium as sugar concentration increased. pH of MOT medium containing fructose was significantly higher than glucose medium and fructose-glucose medium. For fructose medium, pH decreased from 7.4 to 5.9 at concentrations of 2 and 8 percent respectively, while glucose metabolism produced lower pH from 3.8 to 3.2. A combination of fructose and glucose yielded a BC medium with pH going from 4.7 to 3.4 (Fig. 5). Result indicates that glucose was readily converted to gluconic acid production hence the lower pH recorded (3.8<3.4<3.3<3.2). The pathway for fructose metabolism during BC production has been reported [1]. Briefly, fructose is phosphorylated to fructose-6-phosphate and then isomerized to glucose 6-phospahte and further oxidized to gluconic acid. The result in this work is consistent with high acid accumulation reported by [6] or oxidation of d-xylose to xylonic acid [18]. There was significant difference between the type of sugars fermented for BC production and the final pH of the medium [$F(2, 6) = 823.108$, $P<0.05$] on one hand, and the concentrations of the sugars employed [$F(3, 6) = 55.970$, $P<0.05$]. There was however no significant interactions between sugar type and observed final pH during conversion to Biocellulose [$F(6, 24) = 1.977$, $P>0.05$].

Table 1. Summary of regression equation, ANOVA and R^2 values on rate of BC yield'

BC4'=0.972 +0.024. t(5)=0.503,p0.641>0.05; R^2=0.944
BC5'=0.972+0.014. t(5)=8.505,p0.001<0.05; R^2=0.948
BC6'=0.785+0.028. t(5)=2.537,p0.064<0.05; R^2=0.617
BC7'=0.206+0.016. t(5)=0.421,p0.695>0.05; R^2=0.043
BC8'=.-0.113+0.022. t(5)=0-.228,p0.831>0.05; R^2=0.013

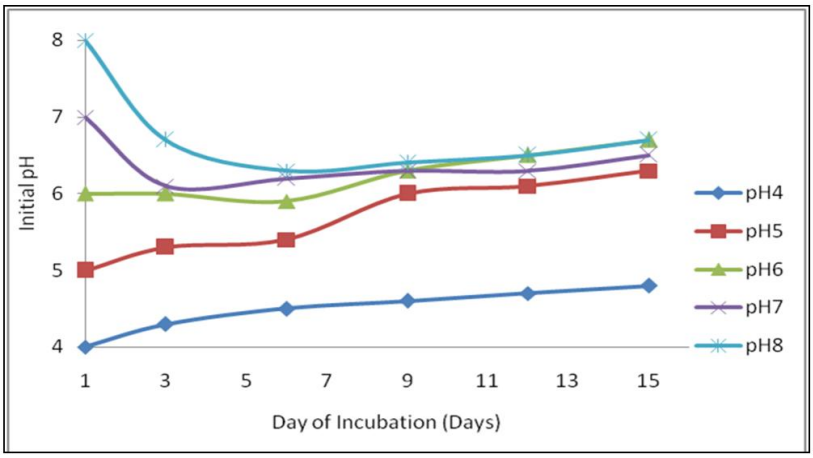

Fig. 4. Changes in pH of medium during biocellulose production

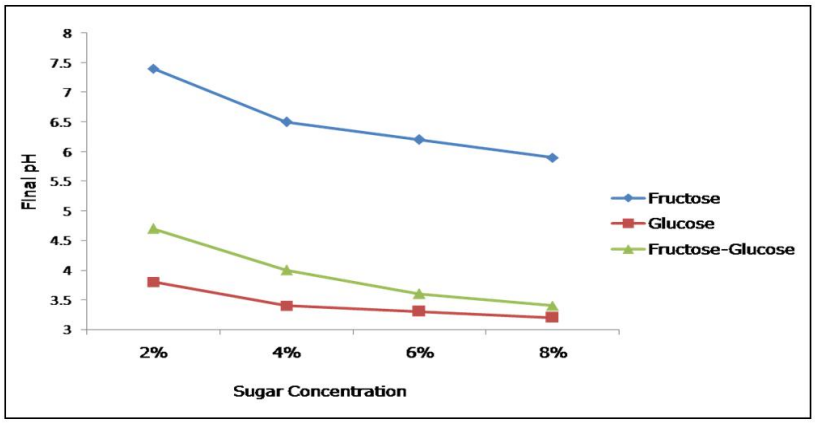

Fig. 5. Effect of sugar concentration on final pH of medium during biocellulose production

6. CONCLUSION

It was established that amount of Biocellulose produced by *Gluconacetobacter xylinus* in *Moringa oleifera* tea–yeast extract broth was affected by pH of the medium. By the end of 15th day incubation, while the maximum BC yield (0.32 g/L) occurred at pH 4 and 5, the lowest (0.01 g/L) was at pH 6. There was no BC at pH 7 and 8. The optimum pH for BC production was therefore 4-6, which was consistent with optimum values in several literatures, in spite of the type of culture medium employed and time of fermentation. The pH changes observed is the result of the metabolism of the sugars added to the medium. *G. xylinus* oxidizes glucose to gluconic acid leading to drop in pH. Thus, while higher initial pH of 7 and 8 were decreasing, lower pH of 4-6 were rising and tending towards neutral or near-neutral pH. Control of pH and sugar concentrations in MOT-yeast extract medium are therefore critical for Biocellulose production.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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