



Production of Bacterial Cellulose from Cellulose Producing Bacteria Isolated from Various Sources like Biofertilizer, Kombucha Scoby using Sugarcane Bagasse as A Low-Cost Carbon Source

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ABSTRACT

Textile industries rely highly on synthetic materials that lead to release of pollutants and waste such as microplastics, dyes, pesticides etc into the environment. Also discarded garments end into landfills contributing to soil and water pollution. Bacterial cellulose can be used in ecological clothing production or they can be used in natural fabrics which is highly pure, biodegradable, biocompatible and it does not contain lignin. In the current study, bacterial cellulose production was attempted from Acetobacter, Rhizobium and an isolate from the Kombucha SCOBY (Symbiotic Culture of Bacteria and Yeast) in the Hestrin Schramm (HS) medium supplemented with glucose with an incubation period of 7 days as a control. Pretreatment of the dry sugarcane bagasse was done with 0.4M acetic acid. The obtained solid fraction was further subjected to enzymatic hydrolysis with partially purified cellulase enzyme produced using Bacillus subtilis. HS medium was supplemented with this sugarcane bagasse hydrolysate. The cellulose pellicles obtained from both HS medium were treated with 0.5% NaOH at 80°C for 15 minutes and then dried for further use. The quantity of cellulose obtained in HS medium supplemented with sugarcane bagasse was significant. This study highlights the benefits of recycling sugarcane bagasse. The bacterial cellulose pellicles obtained can be used in fabric production to reduce the negative impacts of the textile industry on the environment.

KEYWORDS: Bacterial cellulose, sugarcane bagasse, Kombucha SCOBY, cellulase enzyme.

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INTRODUCTION:

The global textile industry, a major contributor to environmental degradation, faces mounting scrutiny due to its dependence on synthetic fibers like polyester and nylon, which are derived from fossil fuels, energy-intensive to produce, and difficult to recycle. The fast fashion model exacerbates these issues, driving massive textile waste and pollution [1]. In response, bacterial cellulose (BC) is emerging as a sustainable alternative with significant promise. Produced by Bacteria such as *Acetobacter* and *Komagataeibacter*. BC is a biodegradable, highly-purity nanofibrous material created through fermentation. Unlike plant cellulose, BC's 3D structure provides strength, flexibility and versatility making it suitable for packaging and electronic applications. Static fermentation yields smooth, leather-like membranes ideal for clothing, while process parameters such as sugar concentration, pH and temperature are critical to optimizing yield and quality [2].

Despite its environmental advantages, the scalability of BC faces economic and technical challenges due to its high production cost involving the use of refined sugar as carbon sources and energy demands for aerobic conditions. To overcome this, researchers are exploring agricultural and industrial waste like sugarcane bagasse hydrolysate and molasses as alternative feedstocks. This approach lowers costs and promotes waste valorisation [3].

However technical barriers such as limited mechanical durability, poor dye uptake, odor retention and microbial susceptibility still hinder issues requires interdisciplinary research in microbiology, material science and textile engineering [4].

In conclusion bacterial cellulose offers a promising path toward more sustainable textiles especially when integrated with circular economy principles and waste-based feedstocks. Continued innovation and

investment are essential to make BC a commercially viable, environmentally responsible alternative in the global textile industry.

MATERIAL AND METHODS

Isolation and screening of cellulose producing isolate from Kombucha SCOBY and biofertilizer:

The cellulose-producing bacteria were isolated from biofertilizer using the standard serial dilution method and plated on GYCA agar medium, then incubated at 30°C for 72 hours. Similarly, cellulose-producing bacteria were isolated from the Kombucha SCOBY using the serial dilution method, plated on GYCA agar, and incubated at 30°C for 72 hours [5].

Gram staining and biochemical tests were performed. The biochemical tests conducted included Indole, Methyl Red, Voges-Proskauer, Simmons' citrate, Urease, Triple Sugar Iron (TSI), Catalase, Oxidase, and Carbohydrate tests [6].

Pretreatment of sugarcane bagasse using acetic acid:

Two different types of sugarcane bagasse (black, green) were sun-dried until all moisture was removed. The dried bagasse was then cut into small pieces and ground into a fine powder using a blender. A total of 250 mL of 0.4 M acetic acid was measured into a 500 mL conical flask, and 5 grams of sugarcane bagasse powder were added. The mixture was treated at 120 °C for 20 minutes. After treatment, the solid fraction was separated by filtration. The recovered solid was dried overnight in a hot air oven at 70 °C, stored in a clean plastic bag, and labeled separately [7].

Screening of *Bacillus subtilis* for Cellulase Production:

Bacillus subtilis was screened for cellulase activity. The strain was inoculated on CMC agar and incubated at 37 °C for 48 hours. Plates were stained with 1% Congo red for 15 minutes, then destained with 1 M NaCl. A clear zone around the streak indicated cellulase production [8].

Production of cellulase enzyme using *Bacillus subtilis*:

200ml of the medium was prepared and *Bacillus subtilis* was inoculated into the medium and incubated at 37°C for 24 hours. The media was centrifuged at 5000rpm for 15 minutes and the supernatant was collected [9].

Ammonium sulphate precipitation and dialysis of Cellulase enzyme:

Cellulase enzyme was precipitated by adding 80% ammonium sulphate to 70 mL of supernatant and incubating at 4 °C for 1 hour. After centrifugation, the pellet was dissolved in phosphate buffer (pH 8.0) and dialyzed for 24 hours at 4 °C [10, 11].

Estimation of cellulase enzyme activity by DNS assay:

Cellulase activity was determined using the DNS assay. A mixture of 900 µL of 1% CMC in 0.02 M phosphate buffer (pH 8) and 100 µL of dialyzed enzyme was incubated at 37 °C for 30 minutes. After adding 3 mL DNS, the reaction was stopped by boiling for 5 minutes. Absorbance was measured at 540 nm. One unit releases 1 µg glucose per minute at 37 °C [11].

$$\text{Cellulase activity U/ml} = \frac{\text{Amount of reducing sugar released } (\mu\text{mol})}{\text{Reaction time (min)} \times \text{Volume of enzyme solution (mL)}}$$

Estimation of the protein content by Lowry et al., method:

5 ml of alkaline solution was added to 1 mL of enzyme and mixed thoroughly. The mixture was allowed to stand at room temperature for 10 minutes. Then, 0.5 mL of diluted Folin-Ciocalteu reagent was added and mixed immediately. After 30 minutes, the optical density was measured at 660 nm using a spectrophotometer, with comparison to a blank and BSA standard curve [12].

Enzymatic hydrolysis of sugarcane bagasse with the partially purified cellulase enzyme:

Enzymatic hydrolysis of sugarcane bagasse was performed using partially purified cellulase enzyme. In a 250 mL flask, 20 g of pretreated bagasse was mixed with 200 mL of 50 mM citrate buffer (pH 5.0) and 1 mL of cellulase. The mixture was agitated at 125 rpm and 50 °C for 48 hours, then centrifuged at 9600 × g for 20 minutes at 4 °C [13].

Estimation of the sugarcane bagasse hydrolysate for reducing sugars with DNS assay

1ml of enzymatic hydrolysate from sugarcane bagasse was mixed with 1 mL of DNS reagent in a clean test tube. The mixture was heated at 100 °C for 10 minutes, then the absorbance was measured at 540 nm using a UV-spectrophotometer [14].

Production of bacterial cellulose in the conventional HS medium as a control

The HS medium was prepared, and *Acetobacter* sp. along with the isolate from Kombucha SCOBY were inoculated into the medium. The cultures were incubated at 30 °C for 7 days under static conditions with intermittent agitation [15] (Shown in Fig. 8, 9).

Production of bacterial cellulose in the sugarcane bagasse hydrolysate supplemented HS medium

The HS medium was prepared using sugarcane bagasse hydrolysate. *Acetobacter* sp. and the isolate from Kombucha SCOBY were inoculated into the medium and incubated at 30 °C for 7 days under static conditions with intermittent agitation [16] (Shown in Fig. 12, 13).

Separation of the bacterial cellulose from the media and purification

The bacterial cellulose pellicle formed at the air–water interface on both media was filtered and treated with 0.5% NaOH for 15 minutes at 80 °C. It was then washed with distilled water, and the wet weight was recorded. The bacterial cellulose was dried overnight, and the dry weight was measured [17].

Kombucha SCOBY isolate sequencing:

Genomic DNA was isolated by adding lysis buffer, lysozyme, and Proteinase K to samples, incubated at 65 °C for 60 minutes, followed by ethanol addition and purification using spin columns. PCR amplification used 16S universal primers with specified cycling conditions. PCR products were analyzed on 1.2% agarose gel.

FTIR and SEM analysis:

Bacterial cellulose from *Acetobacter* sp. and Kombucha SCOBY isolates was examined by SEM for morphology and FTIR using the KBr pellet method for functional group analysis. Samples were gold-coated before SEM imaging, and FTIR spectra were recorded between 4000–400 cm⁻¹, ensuring detailed characterization.

RESULT

Colony morphology:

The Kombucha isolate colonies were round, smooth, creamy white and opaque (as seen in Fig. 1). The colonies of the *Acetobacter* sp. were small, round, mucoid and slightly yellow (as shown in Fig. 2).

Gram staining and biochemical test:

Both the isolates were gram negative rods. Both the isolates were catalase positive and oxidase negative. The biochemical test results for the kombucha isolate were as follows: Indole, MR, VP, urease, TSI negative and citrate positive. The carbohydrate fermentation results are sucrose, dextrose negative, glucose positive and maltose showed gas production and no acid production was observed.

The biochemical test results for the *Acetobacter* sp. were as follows: Indole, MR, VP, urease, TSI negative and citrate positive. The carbohydrate fermentation results are as follows: Glucose, sucrose positive, dextrose negative and acid production was observed in maltose.

Based on the biochemical test the isolate obtained from the *Acetobacter* biofertilizer was identified as *Acetobacter* sp. on comparison with Bergey's manual of determinative Bacteriology.

The isolate obtained from Kombucha SCOBY was identified as *Komagataeibacter xylinus* based on blast analysis in the NCBI blast analysis and phylogenetic tree.

Pretreatment of sugarcane bagasse with acetic acid:

There was a colour change in the pretreated sugarcane bagasse from pale white to light brown.

Screening of *Bacillus subtilis* for Cellulase Production:

A zone of clearance of 1.5cm diameter was observed around the streak line indicating the ability of the isolate to produce cellulase enzyme (as shown in Fig. 3).

Estimation of protein content by Lowry method and enzyme activity by DNS assay in cellulase enzyme:

The cellulase enzyme exhibited significant cellulolytic activity releasing 0.38mmol/ml of glucose during the assay. The enzyme activity was calculated to be 12.67 U/ml (shown in Fig. 5).

A significant colour change was observed indicating the presence of protein. The protein concentration of the cellulase enzyme was determined to be 120 µg/ml (Shown in Fig. 6).

Reducing sugar concentration in the sugarcane bagasse hydrolysate by DNS assay:

By measuring the absorbance of the coloured compound at 540nm using spectrophotometer the concentration of the reducing sugar was determined to be 1.56 mmol/ml for black sugarcane bagasse and 0.55 mmol/ml for green sugarcane bagasse (Shown in Fig. 7).

Production of bacterial cellulose in conventional HS medium as control:

K.xylinus produced bacterial cellulose at a concentration of 90mg/150ml while *Acetobacter* sp. produced 13mg/150ml. This represents an approximate difference of 77 mg between the cellulose production between the two species (As seen in Fig. 10, 11).

Production of bacterial cellulose in HS medium supplemented with sugarcane bagasse hydrolysate:

K.xylinus produced bacterial cellulose at a concentration of 120mg/150ml whereas *Acetobacter* sp. produced 40mg/150ml. This represents an approximate difference of 80 mg in cellulose production. The bacterial cellulose produced using sugarcane hydrolysate supplemented HS medium was found to yield the higher amount of cellulose compared to the conventional HS medium (As seen in Fig. 14, 15).

FTIR analysis of bacterial cellulose produced by *K. xylinus*:

3437.09 cm^{-1} - Broad peak attributed to O-H stretching vibrations of hydrogen-bonded hydroxyl groups in cellulose molecules. 2921 cm^{-1} and 2852 cm^{-1} can be related to the stretching vibrations of C-H present in the cellulose, hemicellulose and lignin structure. 1640.21 cm^{-1} -C-O 6-membered lactone. 1461 cm^{-1} and 1385 cm^{-1} are related to CH₂ bending vibrations. 1210 cm^{-1} is associated with the C-O stretching vibration of the ester group (C-O-C) in cellulose acetate or cellulose triacetate. 1048 cm^{-1} is associated with the C-O-C glycosidic bond stretching vibration, a characteristic feature of cellulose's structure. The broad O-H stretching is a characteristic feature of Cellulose II and is observed between the peaks 3437 cm^{-1} and 2852 cm^{-1} (As shown in Fig. 16).

FTIR analysis of bacterial cellulose produced by *Acetobacter* sp.

3435.76 cm^{-1} - Broad peak attributed to O-H stretching vibrations of hydrogen-bonded hydroxyl groups in cellulose molecules. 2958.74 cm^{-1} - Water soluble ionic cellulose. 2920.43 cm^{-1} - Peak representing C-H stretching vibrations in the glucose units of cellulose. 2850.51 cm^{-1} - symmetric and asymmetric CH stretching in aromatic methoxyl groups. 1643.28 cm^{-1} - Can be attributed to the vibration of water molecules absorbed in cellulose. 1216.70 cm^{-1} - attribute to C-O stretching. 1080.97 cm^{-1} - a peak is associated with C-O stretching vibrations. The broad stretching of O-H bonds is a key feature of Cellulose II. It is identified between the peaks 3435 cm^{-1} and 2920.43 cm^{-1} (As shown in Fig. 17).

SEM analysis of bacterial cellulose produced by *K. xylinus* and *Acetobacter* sp:

The BC produced by *K. xylinus* in HS medium supplemented with sugarcane bagasse hydrolysate exhibited a densely packed structure with thin BC fibers (As seen in Fig. 18).

The BC produced by *Acetobacter* in HS medium supplemented with sugarcane bagasse hydrolysate exhibited a less structurally significant morphology, characterized by less organized and more masses of densely packed fibrils (As seen in Fig. 19).



Fig. 1 GYCA plates showing round white opaque colonies



Fig. 2 Colonies of *Acetobacter* on GYCA agar plates

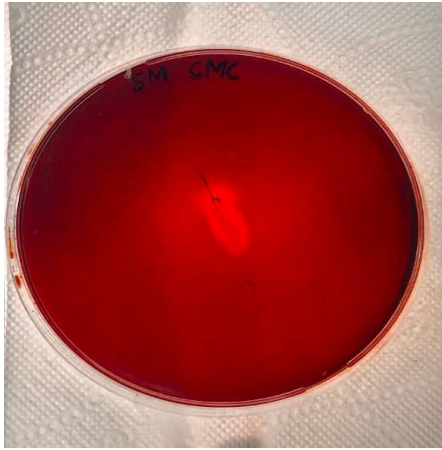


Fig. 3 CMC agar showing zone of clearance

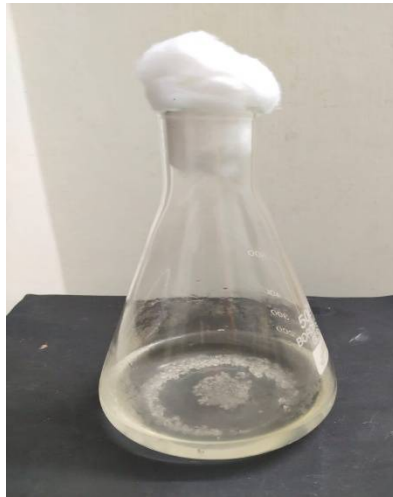


Fig. 4 Cellulase enzyme crystals in enzyme production medium

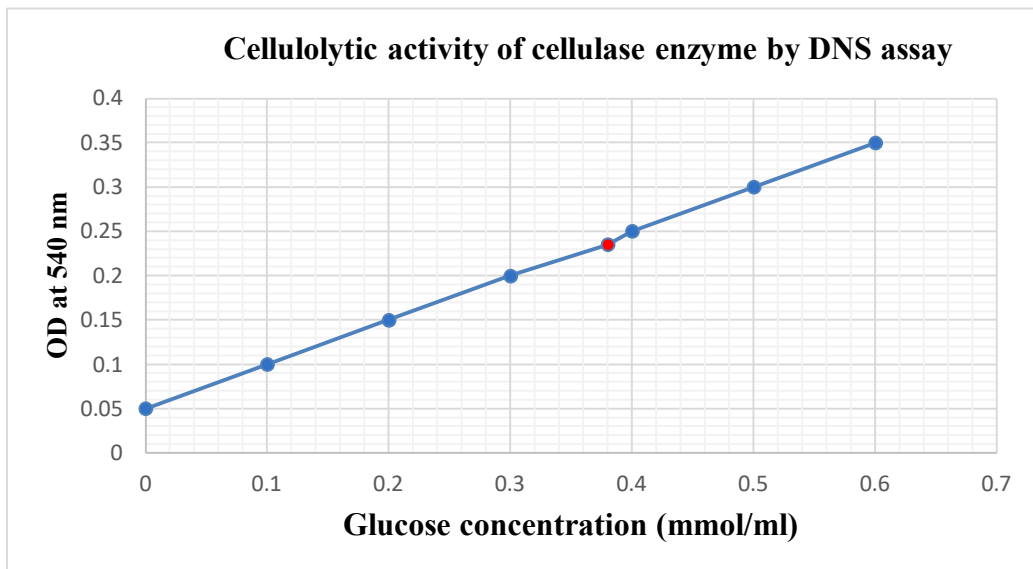


Fig. 5 Estimation of cellulolytic activity of cellulase enzyme by DNS assay

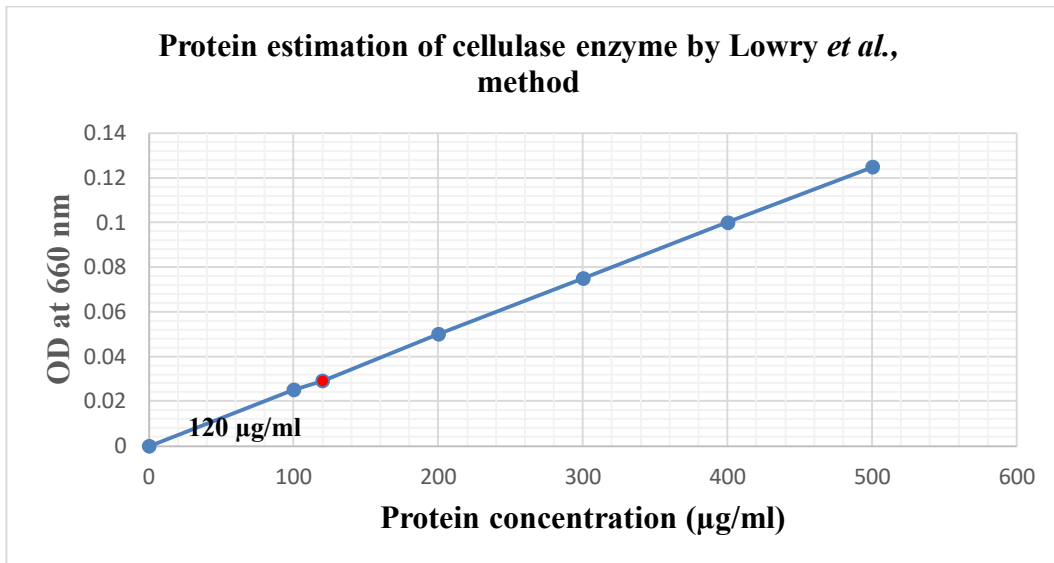


Fig. 6 Estimation of protein concentration in cellulase enzyme by Lowry *et al.*, method

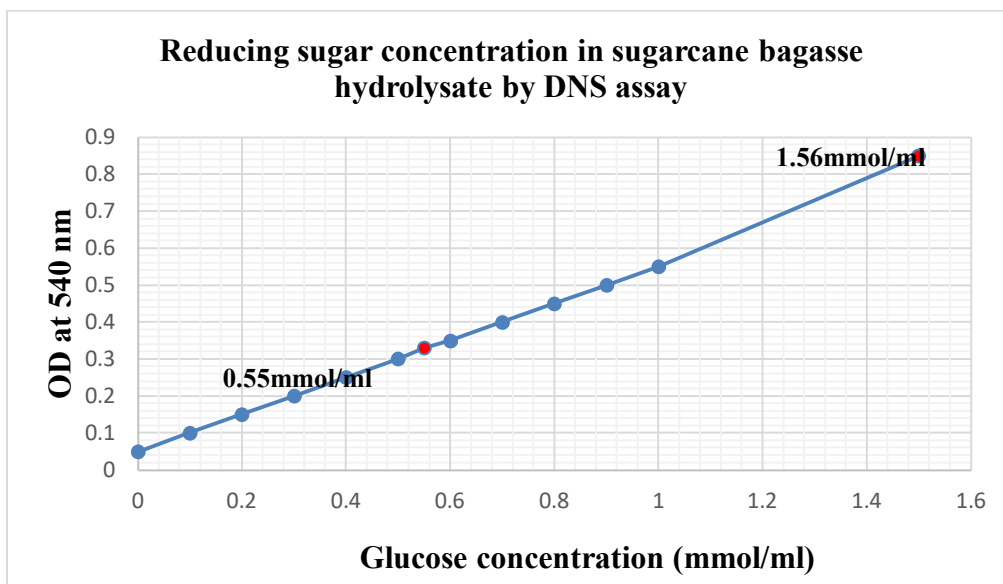


Fig. 7 Estimation of reducing sugars by DNS assay in the sugarcane bagasse hydrolysate

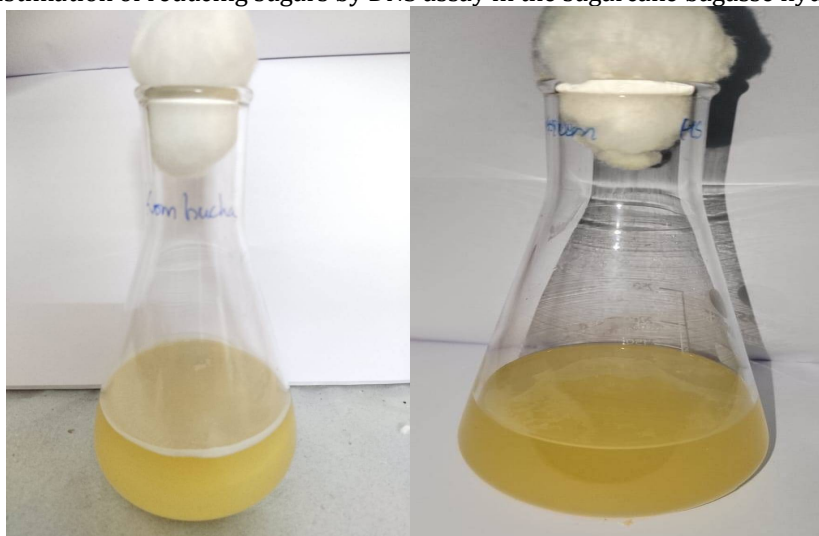
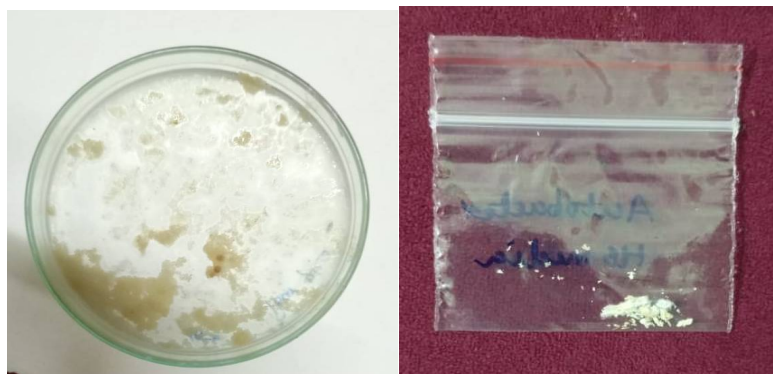


Fig. 8 Production of BC in HS medium with *K. xylinus*

Fig. 9 Production of BC in HS medium with *Acetobacter* sp.



A B
 Fig. 10 BC produced by *K. xylinus* (a) Wet BC (b) Dry BC



A B
 Fig. 11 BC produced by *Acetobacter* sp. (a) Wet BC (b) Dry BC

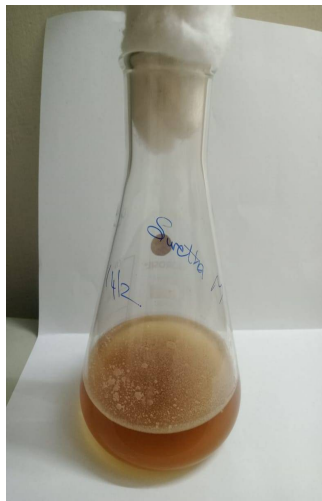


Fig. 12 Production of BC using *K. xylinus*

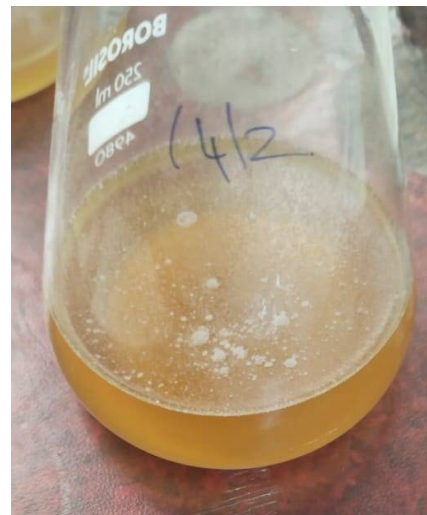


Fig. 13 Production of BC using *Acetobacter* sp.

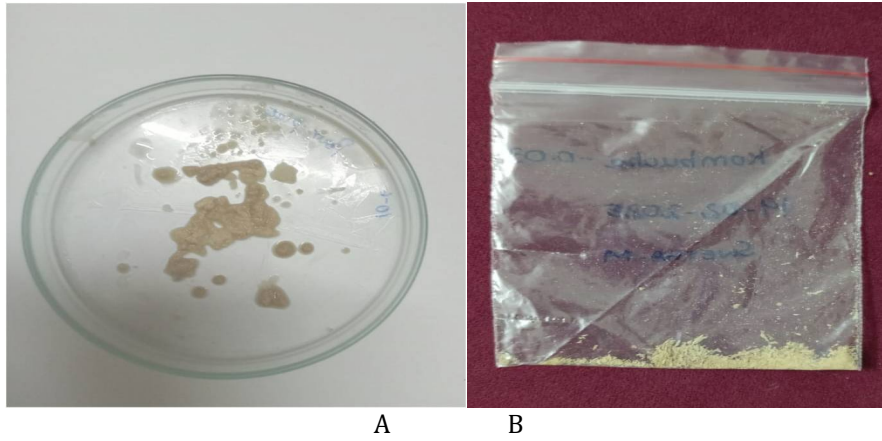


Fig. 14 BC produced by *K. xylinus* in sugarcane bagasse hydrolysate (a) Wet BC (b) Dry BC



Fig. 15 BC produced by *Acetobacter* sp. in sugarcane bagasse hydrolysate (a) Wet BC (b) Dry BC

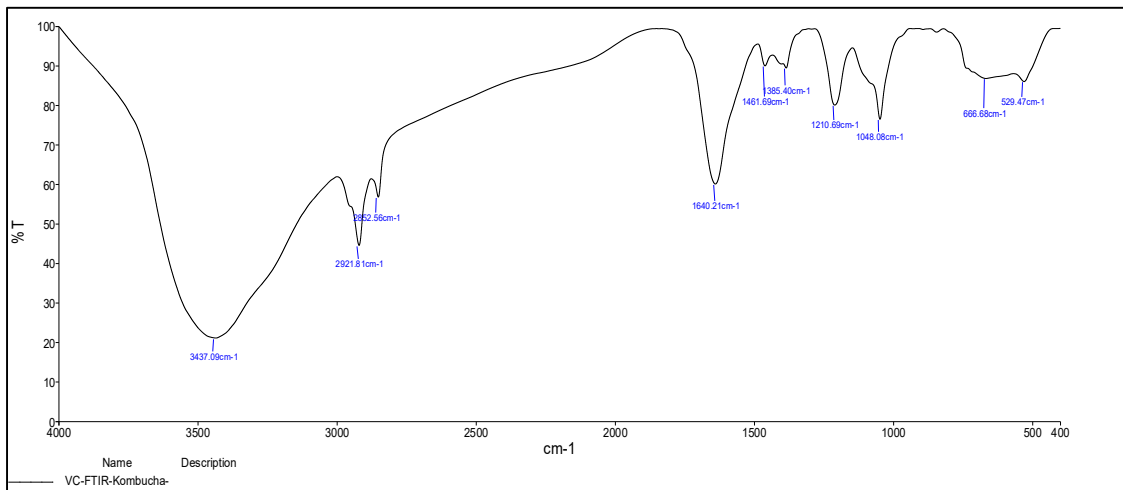


Fig. 16 FTIR analysis of bacterial cellulose produced by *K. xylinus*

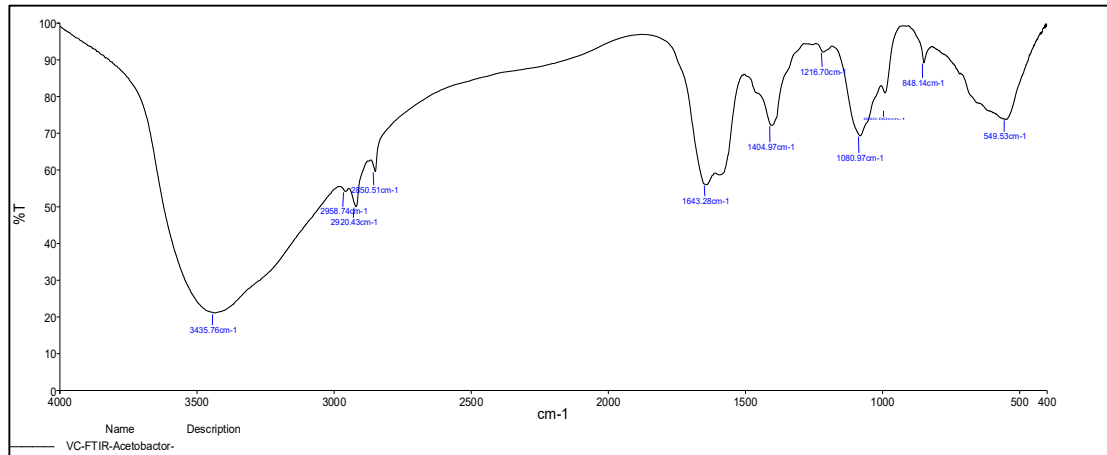


Fig. 17 FTIR analysis of bacterial cellulose produced by *Acetobacter* sp.

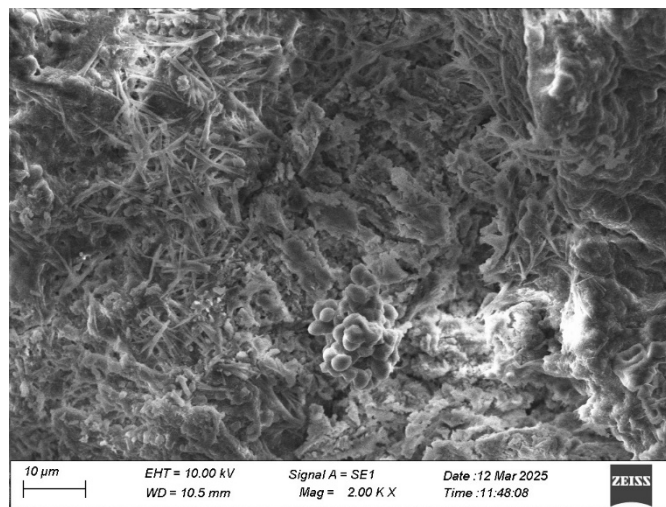


Fig. 18 SEM analysis of BC produced by *Komagataeibacter xylinus*

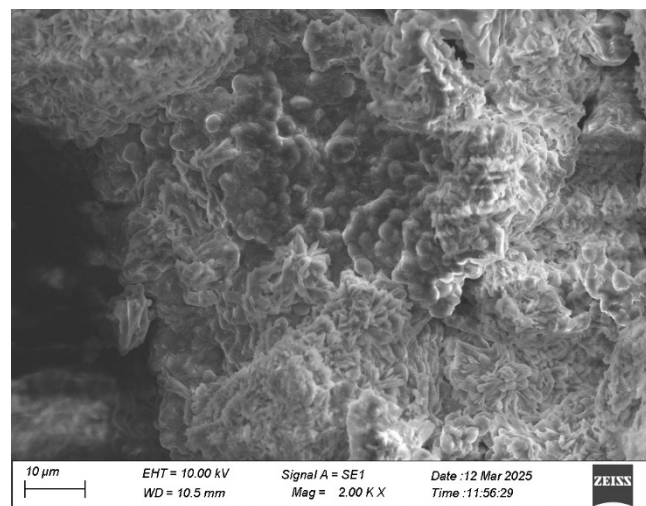


Fig. 19 SEM analysis of BC produced by *Acetobacter* sp.

DISCUSSION

Bacterial cellulose is the common non plant source cellulose which is devoid of other polysaccharides like lignin and hemicellulose. Bacterial cellulose finds application in various pharmaceutical and other industries as an alternative plant cellulose. Among various bacterial genus that can produce bacterial cellulose, *Komagataeibacter* and *Acetobacter* sp. have been reported to produce significant amounts of cellulose. Agricultural residues remain a suitable substrate for different biological processes. Among

various agricultural residues sugarcane bagasse is attractive because of its sugar rich hydrolysate. Utilizing sugarcane bagasse as a substrate will be a means of waste reuse reducing environmental pollution. Pretreatment of sugarcane bagasse by enzymatic hydrolysis results in release of fermentable sugars which can be readily utilized by microorganisms. Hence in the present study pretreated sugarcane bagasse was used as a substrate for bacterial growth and cellulose production. Cellulose producing bacteria was isolated from Biofertilizer and Kombucha SCOBY on GYCA medium.

The bacteria obtained from SCOBY was identified based on biochemical characteristics and molecular techniques-16srRNA study to be as *Komagataeibacter xylinus*. Similar results were obtained from SCOBY isolate by Khiabani et al.,[5]; Chakravorty et al., [18]. The isolate obtained from the biofertilizer was identified based on morphological and biochemical test and was reported to be *Acetobacter* sp. based on Bergey's manual of determinative bacteriology. Black sugarcane bagasse and green sugarcane bagasse were subjected to pretreatment with acetic acid and subsequent enzymatic hydrolysis. The investigation by DNS assay revealed the concentration of the glucose to be 1.56mmol/ml in black sugarcane bagasse and 0.55mmol/ml in green sugarcane bagasse. So according to the results, the glucose concentration being high in black sugarcane bagasse it was chosen as a suitable substrate for Bacterial Cellulose production. There are no earlier studies where comparison between black and green sugarcane bagasse as a substrate for BC production.

The pure culture of *Bacillus subtilis* was screened for cellulolytic activity. The cellulase production was identified by a clear zone around the bacterial colonies on Carboxy Methyl Cellulose Agar (CMC) as reported by Asha et al., [11], Pokhrel et al., [20]. The cellulase producing colonies were inoculated into the sterile enzyme production medium in a conical flask and incubated at 37°C for 48 hours. At the end of fermentation period the medium was 45 centrifuged at 5000 rpm for 15 min and obtained the crude extract. The crude extract was subjected to ammonium sulphate precipitation and dialysis for purification. After purification it was used as an enzyme source for sugarcane bagasse hydrolysis. The purified enzyme was subjected to DNS assay and Lowry et al., method. This result is in accordance with Sethi et al.,[9] who also reported bacterial cellulase production.

The pretreated sugarcane bagasse was hydrolysed with pure cellulase enzyme in citric acid buffer and kept in stirred condition for 48 hours at 37°C to obtain the hydrolysate. Similar works was done by Akintunde et al., [7]. Bacterial cellulose production in HS medium supplemented with sugarcane bagasse hydrolysate was investigated using *Acetobacter* sp. and *K. xylinus* in static condition with intermittent agitation at 30°C for 7days. Both the bacteria showed the ability to utilize the sugarcane bagasse hydrolysate in the fermentation medium leading to the production of bacterial cellulose. There was a significant difference in the yield of bacterial cellulose production by both the strains. Lower yield of bacterial cellulose in conventional HS medium was observed in both the isolates (*Acetobacter* sp. – 13mg/150ml, *K. xylinus* – 90mg/ml) whereas there was a higher yield of bacterial cellulose in the HS medium supplemented with the sugarcane bagasse hydrolysate (*Acetobacter* sp. – 40mg/150ml, *K. xylinus* – 120mg/ml). There was also a significant difference in the bacterial cellulose production between the two isolates. *K. xylinus* produced more bacterial cellulose than *Acetobacter* sp. Our results shown that *K. xylinus* from SCOBY were more efficient in BC production than *Acetobacter* sp. from biofertilizer. According to the previous studies by Khiabani et al., [5], Yamada et al., [19] *Komagataeibacter* has been widely recognized as efficient BC producers.

Finally, the properties of the produced bacterial cellulose were determined using FTIR and SEM. In the earlier studies of Akintunde et al., [7], and Khiabani et al., [5] the spectra of bacterial cellulose produced under various conditions consistently showed characteristic bands of cellulose I, with functional groups identified between 1800 and 500 cm^{-1} . In the current study, FTIR analysis identified characteristic cellulose II bands in bacterial cellulose produced by *Acetobacter* sp. and *Komagataeibacter xylinus*. Functional groups were observed between peaks 3435 cm^{-1} and 2920.43 cm^{-1} in *Acetobacter*, and between 3437 cm^{-1} and 2852 cm^{-1} in *Komagataeibacter xylinus*. The morphology of the bacterial cellulose produced by *Acetobacter* sp. and *Komagataeibacter xylinus* was determined using scanning electron microscopic studies. The result of SEM 46 analysis showed the bacterial cellulose fibrils showing varying diameter range from 2 μm to 10 μm . The results of Bacterial Cellulose production were in agreement with Akintunde et al., [7], Khiabani et al.,[5], Cheng et al.,[16]. The SEM analysis also revealed, the bacterial cellulose produced by the *Acetobacter* sp. is less significant when compared to that of bacterial cellulose produced by *Komagataeibacter xylinus*.

CONCLUSION

The present study was successful in using sugarcane bagasse as a source of carbon in production of bacterial cellulose as a method of reusing agricultural residues and to prevent environmental pollution. Among the two isolates *K. xylinus* produced more bacterial cellulose proving to be a potential source of

bacterial cellulose. After further physico chemical characteristics and thermal characteristics of the isolated bacterial cellulose may find use in various industries. Bacterial cellulose was successfully synthesized using bagasse hydrolysate as the carbon source. Bagasse, being an inexpensive and abundantly available resource globally, significantly reduced the feedstock cost. Consequently, these cost-efficient feedstocks hold great potential as carbon sources for the large-scale industrial production of nanostructured bacterial cellulose materials. This waste recycling model has the potential to contribute significantly to the formulation of sustainable strategies.

Conflict of Interest: The authors declare no conflict of interest.

Ethical Declaration: This study requires no ethical approval.

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Author contribution: Both the authors contributed equally.

Data Availability: No datasets were generated during the study.

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