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Effect of carbon sources on physicochemical properties of bacterial cellulose produced from *Gluconacetobacter xylinus* MTCC 7795

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Abstract: In this study, the effect of modified Hestrin Schramm (HS) medium supplemented with different carbon sources viz., glucose, fructose, galactose and lactic acid on the yield and physicochemical properties of bacterial cellulose (BC) produced from *Gluconacetobacter xylinus* strain MTCC 7795 in shake flask culture conditions was investigated. Growth studies indicated that all carbon sources supported the growth of bacteria, though specific growth rate and doubling time differs. Fructose gave the highest cellulose yield of 7.72 mg/ml after 130 h of fermentation, while yield in glucose and galactose supplemented medium were 4.49 mg/ml and 3.38 mg/ml, respectively. X-ray powder diffraction (XRD) analysis revealed that all BC samples were amorphous in comparison to commercial cellulose. Fourier transform infrared (FTIR) spectroscopic investigations of bacterial cellulose (BC) samples affirm the purity of the cellulose produced. No significant variations in physicochemical properties of cellulose samples produced with different carbon sources were observed. This study for the first time has investigated the effect of carbon sources on physicochemical properties of bacterial cellulose produced by *G. xylinus* MTCC 7795 and provides a strategy for economical production of BC with anticipated application in therapeutics and tissue engineering.

Keywords: bacterial cellulose; biopolymer; FTIR; XRD.

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1 Introduction

Cellulose is the most abundant bio-polymer found on Earth. It contains repeating β-(1, 4) linked glucose subunits and is usually derived from vascular plants (1). Apart from being a notable part of the cell walls of plants, cellulose is also present in algae and fungi, and is alternatively produced by many bacterial species, such as *Sarcina, Agrobacterium, Rhizobium, Acetobacter, Achromobacter, Aerobacter, Azotobacter, Salmonella* and *Escherichia*. *Acetobacter xylinum* is one of the most studied strains and has been known to produce pure cellulose, for more than 200 years. It was primarily isolated from Nata de Coco, a desert of the Philippines (2).

However, growing industrial demand of cellulose and cellulose based products is putting a negative pressure on the ecosystem, creating a negative ecological imbalance (3, 4). Thereby, emphasis has been put upon the production of cellulose from alternative sources. A great deal of interest has been created worldwide on the production of cellulose using these alternative sources, to keep the environmental impact to a minimum (5). Existing reports show that bacterial cellulose (BC) has proven to be the most promising approach for cellulose production from an alternative source (6).

BC possess superior physicochemical properties such as higher purity, crystallinity, biocompatibility and moisture retention compared to plant cellulose (7, 8). Owing to its unique properties, BC has found application in a number of fields such as the paper and textile industry (7), diaphragms for electro-acoustic transducers, the food industry (9, 10), film coatings as drug delivery systems (11), pharmaceuticals and cosmetics, optically transparent composites (12, 13), substrates for OLEDs (13), biomaterials in cosmetics and medicine (11, 14–16). In recent years, biomedical applications of BC have received considerable attention in the literature, particulary in wound dressing, blood vessels, vascular grafts and delivery systems of drugs and proteins (17). Various studies have proven the application of BC scaffolds in the field of tissue engineering and wound healing (15). Saska et al. (2011) reported

that BC composites containing hydroxyapatite showed bone regeneration properties and could ideally be used as bone grafts (16). Such versatile applications obtrude BC as one of the most important environment friendly biopolymers of choice and increase its demand.

Despite the diverse applications of BC, there are many bioprocess problems associated with its production such as low yield and high cost (18). To overcome these limitations, there has been extensive research on the enhanced production of BC from *A. xylinus*, by optimization of bioprocess conditions in order to achieve high yields to meet the worldwide demand for cellulose. New technologies involving the use of industrial waste as a carbon source for BC production promotes economic advantages. Agroindustrial byproducts such as molasses (19), konjac powder hydrolysate (20), and various fruit juices (21) have been successfully utilized for low cost BC production. Yang et al. (2013) used elephant grass acid hydrolysate as the substrate for production of BC which resulted into a yield of 6.4 g/l at a production cost which was more cost effective than the conventional substrate (22). While, Wu and Liu (2012) have documented the production of bacterial cellulose from distillery waste water from the winery industry, which resulted in a 50% higher yield at a production cost of almost 67% less than the conventional medium (23).

The aim of this study was to determine the effect of carbon sources on cellulose yield by *Gluconacetobacter xylinus* MTCC 7795. BC samples produced were characterized for their physiochemical properties and purity for its anticipated application as drug delivery systems and transdermal patches.

2 Materials and methods

2.1 Bacterial strains and culture conditions

Gluconacetobacter xylinus MTCC 7795, used in this study, was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The bacterial strain was cultivated in Hestrin-Schramm (HS) medium (24). The cell suspension was cryopreserved in 20% glycerol at -80°C. For culture revival, 100 µl of cell suspension stored as glycerol stock was added to 50 ml of revival medium (HS) and incubated at 30°C, 120 rpm in a shaker incubator.

2.2 Medium composition and cultivation

All chemicals used were of analytical grade, unless specified and were commercially available. Cell growth and BC production was evaluated in a modified HS supplemented with 2% different carbon sources (glucose, galactose, fructose, lactic acid). An active growing culture was maintained in HS medium having 2% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.27% (w/v) disodium phosphate, 0.15% (w/v) citric acid, 2% (w/v) agar (pH 6.0). Further, a primary inoculum was prepared by transferring a single bacterial colony from an actively growing culture into 10 ml of each modified HS medium. Cell growth studies and BC production studies were evaluated in 100 ml different modified HS media inoculated with 10% (v/v) primary inoculums, at 30°C and 120 rpm in a shaker incubator.

3 Analytical methods

The culture broth (1 ml) was collected at different time intervals from all media and was analyzed for BC yield and bacterial growth.

3.1 Recovery of bacterial cellulose

BC was recovered from the culture broth using the alkali treatment method (6, 25). Briefly, the broth was centrifuged at 3075 ×*g* at room temperature for 10–15 min. Supernatant was discarded and pellet was given alkali wash with 2% NaOH solution. The pellet was boiled in NaOH solution for 90 min. This NaOH treatment was repeated three times and then, the solution was centrifuged again at 3075 ×*g* at room temperature for 20 min. Cellulose was dried in an oven at 80°C overnight.

3.2 Analysis of bacterial cellulose

Culture broth was treated with 1U cellulase (Maps Enzymes Limited, Gujarat, India) prior to cell growth estimation. Bacterial cell growth was estimated by measuring optical density at 600 nm and was expressed as specific growth rate (h^{-1}). BC was estimated using phenol sulfuric acid (26) method with glucose as standard.

3.3 XRD

BC samples recovered were crushed to fine powder and were placed in X-ray holder. X-ray diffraction spectra were recorded using Cu-Kα radiations on X-ray diffractometer (Shimadzu 6000 XRD, Japan). The voltage and current used were 40 kV and 30 mA, respectively at room temperature. Scans were performed over 2θ=5–70° range with a scan speed of 2 deg/min to identify the change in the crystal structure of BC samples.

3.4 Fourier transform infrared (FTIR) spectroscopy

BC was characterized using FTIR spectroscopy (Spectrum BX-II spectrophotometer, Perkin Elmer, Germany). Sample pellets were prepared by mixing equal ratios of BC with spectroscopic grade KBr [Sisco Research Laboratories (SRL) chemicals, India] (1:100) and subsequently pelletizing the mixture at 10 Torr. FTIR data in absorption mode was recorded with 2 cm^{-1} resolution in the wave number range of 4000–400 cm^{-1}. Commercially available microcrystalline cellulose (CDH, India) was used as standard.

3.5 Statistical analysis

All experiments were carried out in triplicates and results were expressed as a mean of triplicate measurements with error bars depicting standard errors.

4 Results and discussion

4.1 Effect of carbon sources on cell growth and BC production

Bacterial cellulose production by *G. xylinus* MTCC 7795 was investigated with culture media enriched with various carbon sources. Results showed variations in specific growth rate and doubling time of *G. xylinus* MTCC 7795 with different carbon sources (Table 1). Specific growth rate of bacteria was calculated as reported previously (27, 28) using Eq. 1.

$$N_t = N_0 e^{\mu(t_2 - t_1)} \quad [1]$$

Where, N_t and N_0 are the number of bacterial cells/ml during the exponential phase of cell growth, at time t_2 and t_1, respectively. μ represents the specific growth rate of bacterial cells under the defined culture conditions. It is also related to doubling time (t_d) of an organism by Eq. (2) (27, 28). Doubling time of bacteria was calculated by Eq. 2.

Table 1: Effect of different carbon sources on specific growth rate (μ) and doubling time of *G. xylinus* MTCC 7795 in modified HS medium.

Carbon source	Specific growth rate (μ)	Doubling time (h^{-1})
Glucose	0.47	1.46
Fructose	0.47	1.47
Lactic acid	0.14	4.95
Galactose	0.61	1.13

$$t_d = \frac{0.693}{\mu} \quad [2]$$

The growth studies of bacteria were carried out for 130 h in culture media supplemented with various carbon sources (Figure 1). After 130 h of incubation, all carbon sources supported the growth of bacteria. The amount of BC produced was estimated during 130 h of cultivation. Results showed that the yield of BC in HS medium supplemented with fructose was higher (7.72 mg/ml) than medium supplemented glucose galactose and lactic acid with BC yield of 4.49 mg/ml, 3.38 mg/ml and no yield, respectively (Figure 2). BC samples obtained from media supplemented with fructose and glucose were further characterized for their physicochemical properties and were designated as BC1 and BC2, respectively. Results are in agreement with the previous reports where effect of carbon sources including monosaccharides, oligosaccharides, organic acids, sugars on enhanced production of bacterial cellulose has been investigated (29–31). Present study highlights the ability of *G. xylinus* MTCC 7795 to metabolize a number of carbon sources and their effect on cellulose production.

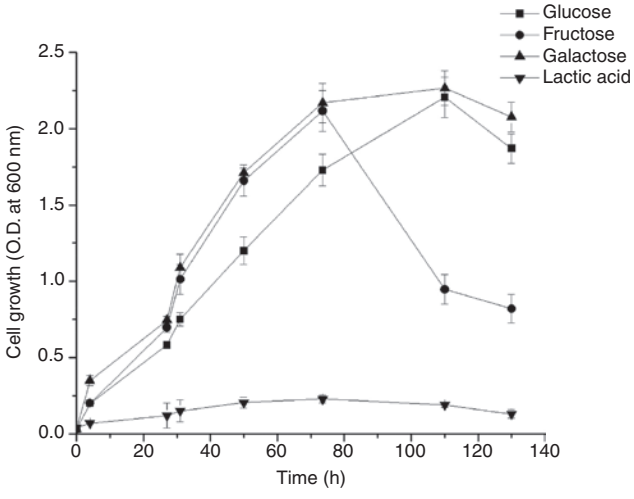

Figure 1: Growth studies of *G. xylinus* MTCC 7795 in modified HS medium with different carbon sources.

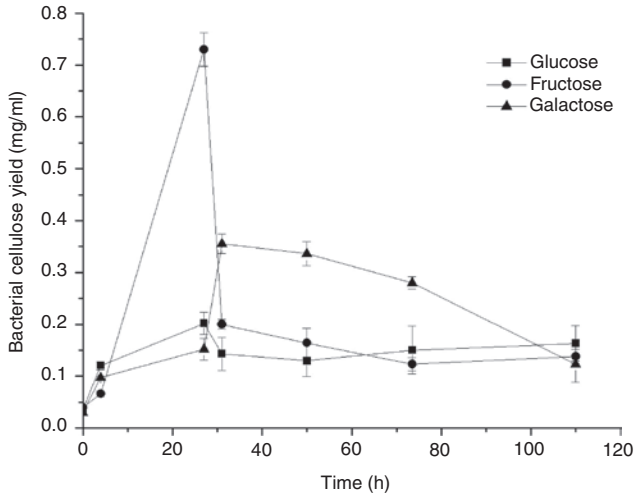

Figure 2: Bacterial cellulose production by *G. xylinus* MTCC 7795 during the course of fermentation using different carbon sources.

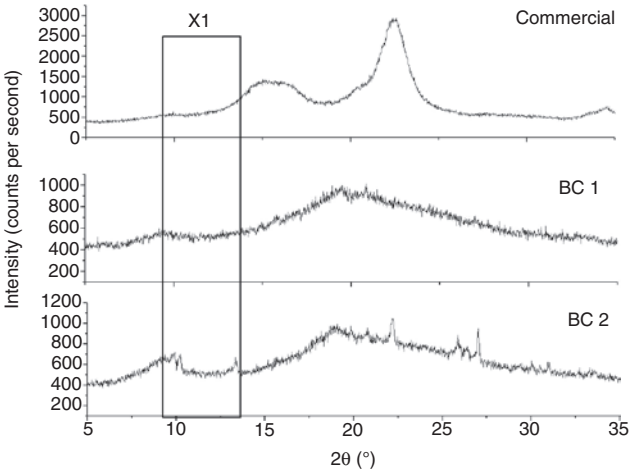

Figure 3: X-ray diffractograms of commercial cellulose, BC1 and BC2 samples.

The strain readily utilized glucose, fructose and galactose for cellulose production during 5 days of the fermentation period and resulted in BC production (Figure 2). Glucose, fructose and galactose have been reported to transport through the bacterial cell membrane and assimilate into the cellulose biosynthetic pathway (32, 33). The low yield obtained with galactose can be explained by the fact that galactose uptake from medium was inefficient and it could not be transported across the cell membrane (31). Results are in good agreement with the findings of Mikkelsen et al. (2009), suggesting the effect of carbon sources on bacterial growth rate and cellulose production. To the best of our knowledge, no other publication has investigated the effect of carbon sources on physicochemical properties of cellulose produced by *G. xylinus* MTCC 7795.

4.2 XRD

The X-ray powder diffraction (XRD) patterns of BC samples and commercial cellulose are displayed in Figure 3. Commercial cellulose showed prominent peaks at 14.59° and 22.92°, corresponding to the crystallographic planes of (101) and (002), respectively. The X-ray diffractograms of prepared BC samples revealed their amorphous nature. The amorphous character is evident through the absence or strong reduction of the (101), (101) and (002) peaks, corresponding to characteristic Bragg angle values for cellulose I (34). As seen in Figure 3, superposition of these peaks due to less crystallinity of BC has taken place. More amorphous nature of BC samples is also evident at position X1, which becomes more pronounced (more intense and broad). This phenomenon can be explained by reduction in the intra- and intermolecular hydrogen bonding that might occur during the transformation of cellulose I into amorphous cellulose (34). In order to further substantiate the data obtained from XRD, we performed FTIR characterization to examine the fundamental vibrational modes of the constituents groups, existing in BC1 and BC 2.

4.3 FTIR

The positions and intensities of infrared absorption bands as obtained from FTIR spectroscopic investigations, are characteristic of a particular compound and can be used to identify the BC by comparing it with commercial cellulose (CDH, India) (Figure 4A–C). Cellulose is reported to exist in the form of two allomorphs, cellulose I and cellulose II. A dominant form in nature, cellulose I is microfibrillar crystalline array of linear β-1,4-glucan chains which are aligned parallel to one another with the same polarity (35).

Acquired FTIR spectra showed that the characteristic vibrational modes of prepared BC produced were almost in the same fingerprint regions as reported earlier (6, 36). Comparison of FTIR data of BC samples with commercial cellulose in the wave number range of 4000–400 cm^{-1} revealed the presence of a characteristic vibrational peak of C-H out of the plane bending at 836–808 cm^{-1} (Figure 4A–C). In Figure 1B and C, presence of absorption band around 1050 cm^{-1}, corresponds to C-C bonds of the monosaccharide units of cellulose and is generally referred to another signature band of cellulose. The FTIR absorption band at 888 cm^{-1} in commercial cellulose, assigned to C-O-C stretching at β, (1–4) linkage, is designated as an "amorphous"

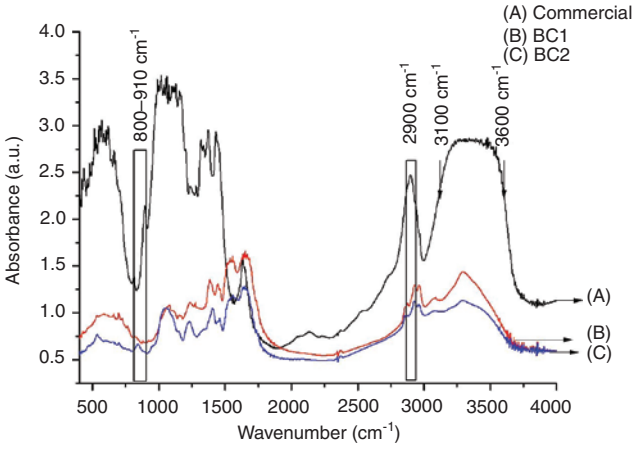

Figure 4: FTIR spectra of (A) commercial cellulose, (B) BC1, (C) BC2.

absorption band. Slight shifting of band towards higher wave number (872–868 cm^{-1}) with decreased intensity has been observed for BC samples. FTIR spectroscopic data exhibits a weak band in the range of 850–808 cm^{-1} and a strong band at 1430 cm^{-1}, corresponding to cellulose allomorph as cellulose I (37). Figure 4B and C showed bands at 1036–1044 cm^{-1}, depicting the characteristic bending of C-O-H bond of carbohydrates. The appearance of a broad band in the wave number region of 3600–3100 cm^{-1} was assigned to OH stretching modes and thus provides considerable information about the presence of hydroxyl groups in bacterial cellulose samples. Findings by Ciolacu et al. 2011 suggested the existence of intramolecular hydrogen bonds of O(2)H···O(6) and O(3)H···O(5), and the intermolecular hydrogen bonding of O(6)H···O(3) in cellulose samples in the range of 3455–3410, 3375–3340 and 3310–3230 cm^{-1}, respectively. The existence of a CH_2 bending mode at 1430 cm^{-1} has been correlated with the degree of crystallinity and is referred to as "crystallinity band" (34). We measured the crystallinity of cellulose samples by estimation of ratio of crystallinity (CR), as shown in Table 2. The ratio of crystallinity (Cr.R1 and Cr.R2) was determined through methods adopted by (34) where Cr.R1 is absorbance ratios from 1372 to 2900 cm^{-1} and Cr.R2 is absorbance ratio from 1430 to 893 cm^{-1}. Data obtained from crystallinity ration Cr.R1 showed that crystallinity of samples BC1 and BC2 decreased significantly in comparison to commercial cellulose, suggesting that BC samples were amorphous in nature. Higher absorbance at 893 cm^{-1}, as observed for commercial cellulose, resulted in decrease of Cr.R2, in comparison to BC1 and BC2. Data obtained from crystallinity ratios suggested prepared BC samples to be amorphous, as compared to commercial cellulose. No significant variation in the crystallinity of BC 1 and BC 2 could be observed from FTIR analysis. The absence of any non characteristic peak in BC samples in comparison to commercial cellulose affirms the purity of prepared BC samples and therefore, paves the way for its application in various fields (38).

Table 2: Ratio of crystallinity index (Cr.R) of different cellulose samples at FTIR fingerprint regions.

Sample	Cr.R1[a]	Cr.R2[b]
Commercial cellulose	1.19	0.99
BC1	0.99	1.79
BC2	0.89	1.52

[a]Absorbance ratio (A_{1372}/A_{2900}).
[b]Absorbance ratio (A_{1430}/A_{893}).

Studies performed by Mikkelsen (31) revealed no significant changes in molecular and microscopic features of bacterial cellulose produced by *G. xylinus* ATCC 5324 with various carbon sources (glucose, fructose, mannitol, glycerol, sucrose and galactose).

5 Conclusion

Fermentative studies with *G. xylinus* MTCC 7795 for BC production demonstrated the effect of various carbon sources on the specific growth rate and cellulose production profile. The study has proposed that no significant variations in the physicochemical properties of bacterial cellulose produced with different carbon sources were observed. The availability of limited reports on bacterial cellulose production by *G. xylinus* MTCC 7795 enhances the essence of this study in understanding the effect of process conditions on BC characteristics. The study can be further extended for optimization of process conditions for enhanced production of BC and its anticipated applications in therapeutics and tissue engineering.

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