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Sustainable Approach for Green Synthesis of Bacterial Cellulose from *Acetobacter Xylinum* Ncim 2526

Madhur Kumar Singh*[1], Ashutosh Pandey[1], Annika Singh[2], Mukesh Kr. Singh[1]

[1]*Uttar Pradesh Textile Technology Institute Kanpur India*

[2]*Institute of Biosciences and Biotechnology, CSJMU Kanpur*

*Corresponding Author: mksingh9129489091@gmail.com

Abstract:

Bacterial cellulose (BC), a versatile biopolymer produced by *Acetobacter xylinum* NCIM 2526, was synthesized and systematically characterized to evaluate its morphological and water-holding properties. Cultivation was carried out in Hestrin–Schramm medium using both shaking and static conditions to optimize yield and structural characteristics. Sub-culturing resulted in pellicle formation (4–6 mm), which was further developed into larger pellicles (12–20 mm) and membranes with a thickness of 1.5 mm. Post-synthesis purification was achieved using 0.1 N NaOH, followed by sequential hot and cold washing and drying. Morphological analysis by scanning electron microscopy (SEM) revealed ribbon-like microfibrils with an average diameter of 4.7 µm in pellicles, arranged in a three-dimensional network. Membranes displayed a highly interconnected porous microstructure with uniform pore distribution across the cross-section, enabling superior mechanical stability. This unique nanofibrillar arrangement directly influenced the water-holding capacity, as the porous and interlinked microfibrils facilitated high moisture absorption and retention. Results demonstrate that bacterial cellulose produced under optimized conditions exhibits exceptional morphological uniformity and water-holding potential, indicating its suitability for applications in biomedical scaffolds, wound dressings, filtration systems, and food packaging.

Keywords:

Bacterial Cellulose, Acetobacter Xylinum, Scanning Electron Microscopy, X-ray Diffraction, Differential Scanning Calorimeter, and Thermal Gravimetric Analyser.

Introduction

Cellulose is the most abundant natural polymer on Earth, forming the fundamental structural component of plant cell walls and playing an essential role in the global carbon cycle. Its biocompatibility, biodegradability, and renewability have made cellulose a cornerstone material in the development of green and sustainable products for a wide range of industrial sectors including textiles, paper, packaging, composites, biomedical materials, and electronic devices [1-3]. However, plant-derived cellulose often contains lignin, hemicellulose, and other impurities that require extensive chemical treatments for purification, leading to high processing costs and environmental burdens. In contrast, bacterial cellulose (BC), synthesized extracellularly by species of *Komagataeibacter* (previously *Acetobacter xylinum*), represents a highly pure, nanoscale form of cellulose that requires no intensive chemical purification. BC is typically produced in Hestrin–Schramm (HS) medium, a glucose and nitrogen-rich broth originally formulated in 1954, under static or agitated fermentation conditions. Production can also be optimized through medium modifications, carbon source variation, and pH adjustments, which strongly influence yield and microstructural characteristics [4-7]. Previous studies have confirmed that carbon source plays a decisive role in yield. For example, Embuscado et al. (1994) [8] demonstrated that glucose-based HS medium produced the lowest yield (1–1.5 g/L), whereas fructose and fructose–sucrose mixtures significantly enhanced productivity, reaching up to 7.38 g/L. Similarly, Ramana et al. (2000) [9] showed that sucrose and mannitol, coupled with peptone or casein hydrolysate, supported higher BC

productivity compared to other sugars such as galactose, maltose, or starch, which yielded less than 2.0 g/L. Additional studies indicated that sucrose and glycerol were especially effective carbon substrates, achieving yields of 3.83 g/L and 3.75 g/L, respectively (Mikkelsen et al., 2009) [10]. Nitrogen source and pH also exerted significant effects, with yeast extract and peptone combinations producing better yields, while acidic conditions around pH 4.5 were more favourable than neutral ranges (Embuscado et al., 1994) [8]. These findings illustrate the complex interplay between carbon to nitrogen ratio, pH, and culture conditions in optimizing BC production as shown in previous studies.

The remarkable physicochemical properties of BC, including ultrafine fiber network, high crystallinity, remarkable tensile strength, and superior water retention, have led to its adoption in a variety of sectors. In the biomedical field, BC has been applied in wound dressings, artificial skin, blood vessel substitutes, and drug delivery systems (Czaja et al., 2006; Czaja et al., 2007; Hong et al., 2012) [11-13]. Its biocompatibility allows direct integration into human tissues, while its ability to form composites with polymers such as gelatin or chitosan enhances its functionality (Ciechanska et al., 2004) [14]. In addition, BC has shown potential in food technology as a stabilizer and texture modifier, in acoustics and electronics due to its nanostructured fibril arrangement, and in packaging and water purification applications (Janpetch et al., 2016; Chatchawanwirote et al., 2019) [15-16]. Nonetheless, the widespread industrial adoption of BC remains restricted by limitations such as relatively low yield in conventional HS medium, long fermentation times, and the high cost of refined carbon and nitrogen sources. For example, Son et al. (2001) [6] reported yields of only 3.8 g/L under optimized HS medium conditions with fructose–sucrose mixtures, while other studies achieved even lower yields below 5 g/L (Mikkelsen et al., 2009; Zhang et al., 2016) [10,17]. Although modified HS medium has led to significant improvements in yield, Ruka et al. (2012) [18] and Hu et al. (2010) [19] reported 10 g/L by supplementing corn steep liquor, and Hong et al. (2012) [13] achieved Maximum BC yield using ethanol extracts, the economic feasibility remains challenging for large-scale processes. Thus, despite its superior material properties and versatility, bacterial cellulose requires process innovation and cost-effective synthesis strategies to become commercially viable in diverse industries.

To address this issue, extensive research has been carried out to lower production costs and improve yields, primarily through the replacement of expensive refined sugars and nitrogen sources with agro-industrial by-products and waste materials. Revin et al. (2018) [20] demonstrated that acidic food industry by-products such as wheat thin stillage and whey could triple BC production compared to HS medium, yielding 6.19 g/L and 5.45 g/L, respectively. Similarly, Tsouko et al. (2015) [21] successfully utilized pineapple peel and sugarcane juice as alternative carbon sources, producing up to 2.8 g/L BC, which exceeded the yield from HS medium (2.1 g/L). Agricultural residues have also been employed, such as coffee cherry husk extract enriched with corn steep liquor, urea, and acetic acid, which resulted in 5.6–8.2 g/L of BC, more than threefold the control medium (Rani et al., 2013) [22]. Moosavi-Nasab et al. (2011) [23] explored low-quality date syrup as a carbon source, achieving 4.35 g/100 ml, while Treesuppharat et al. (2017) [24] reported that enzymatic hydrolysis of cotton waste textiles in ionic liquid media yielded 10.8 g/L, significantly outperforming glucose-based media. Other studies have improved productivity by optimizing culture conditions rather than replacing the medium. For instance, Zhang et al. (2016) [17] demonstrated that altering the carbon to nitrogen ratio improved yields up to 2.76 g/L, while Mohammadkazemi et al. (2015) [25] obtained 4.77 g/L by introducing ethanol into HS medium under shaking conditions. Taken together, these investigations highlight the dual strategy of cost reduction. The first is lowering medium costs through substitution with low-cost raw materials. The second is improving productivity through culture optimization.

Building upon this foundation, the present study investigates the cost-effective synthesis of bacterial cellulose using Hestrin–Schramm medium under optimized shaking and transfer conditions. Specifically, 150 ml of HS medium was employed in a two-step process. In the first step, BC was cultivated at 110 rpm shaking frequency to maintain a uniform culture broth. In the second step, a small BC sphere pellicle formed during the first phase was transferred into fresh medium, which supported enhanced cellulose biosynthesis. The novelty of this study is to opted sustainable approach to enhance the BC production yield by using Hestrin-Schramm medium.

Materials and Methods

Experimental Preparation

The bacterial strain *Acetobacter xylinum* NCIM 2526 was procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. All media ingredients including glucose, peptone, yeast extract, disodium phosphate, hydrochloric acid, sodium hydroxide, D-sorbitol, and agar were of analytical grade and supplied by SRL Chemicals.

In this study, bacterial cellulose (BC) was produced in two successive stages involving both shaking and static culture conditions. Initially, sub-culturing was performed by transferring a 3 mm colony of *A. xylinum* NCIM 2526 from an agar plate into 150 ml of standard Hestrin–Schramm (HS) medium [8]. The inoculated flasks were incubated at 30 °C under shaking at 110 rpm for 3 days, resulting in the formation of small BC pellicles (4–6 mm in diameter). Subsequently, one pellicle from the subculture was transferred into 150 ml of fresh HS medium and incubated under a combined regime of 4 days shaking at 28 °C followed by 3 days static cultivation. This method facilitated the growth of large pellicles (12–20 mm) along with a continuous BC membrane of approximately 1.5 mm thickness.

The harvested BC pellicles and membranes were purified by treatment with 0.1 N NaOH to remove bacterial cells and residual medium components. Post-treatment, the samples were subjected to two cycles of hot washing and two cycles of cold washing until neutral pH was attained. Finally, the purified cellulose was dried in a hot air oven at 50 °C for 4 hours to obtain dried BC pellicles and membranes suitable for further analysis.

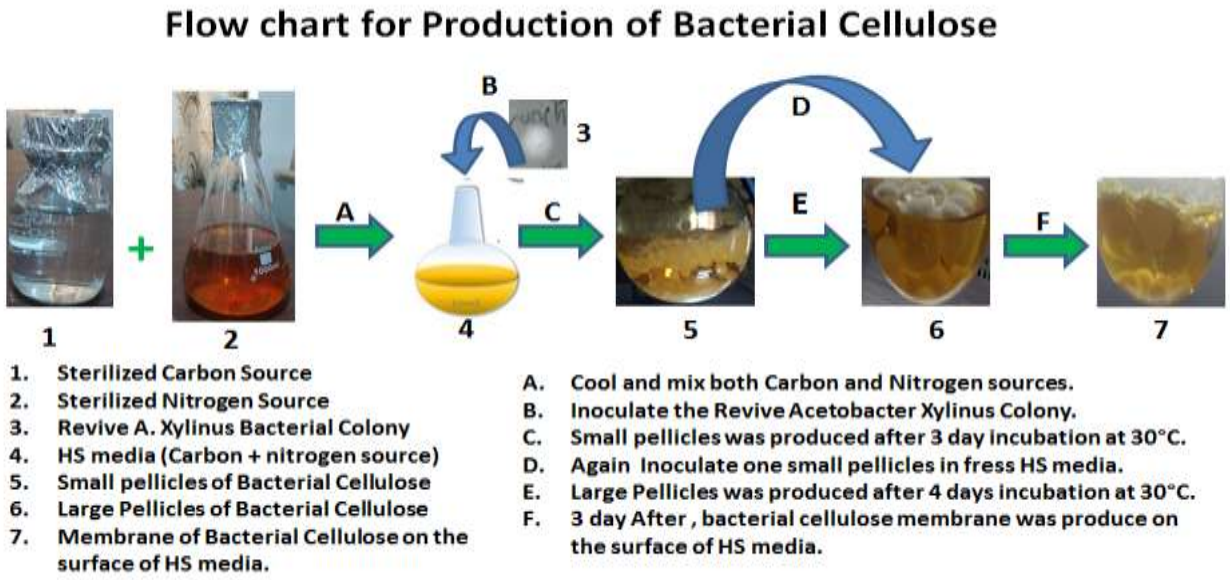

Fig.1. Flow diagram of bacterial cellulose production in HS media.

Characterization

The purity and structural properties of bacterial cellulose (BC) powder were evaluated using Fourier Transform Infrared (FTIR) spectroscopy. FTIR spectra of virgin BC were recorded in the wavenumber range of 4000–100 cm^{-1} in transmission mode with an attenuated total reflection (ATR) accessory using an Alpha-model FTIR spectrometer (Bruker, Germany).

The morphological features of gold-coated (30 s sputtering) BC pellicles and membranes were examined by scanning electron microscopy (SEM) using a benchtop JEOL JCM-7000 model microscope.

The crystalline structure was determined by X-ray diffraction (XRD) analysis using a multipurpose Empyrean diffractometer (PANalytical, Netherlands). Both the crystallinity index and crystallite size were calculated, and

the data were processed using the HighScore software package integrated with the XRD system. The methodology followed was consistent with the procedures described in previous studies [4-5].

Water holding capacity (WHC) of BC was determined using a digital moisture meter (Model 101). The WHC was calculated using the following equation:

$$\text{Water holding ability} = \frac{\text{Wt. of moist BC} - \text{Wt. of oven dry BC}}{\text{Wt. of oven dry BC}} \text{X100}$$

Result and Discussion:

The purpose of this study to know the effect of incubation conditions on bacterial cellulose physical and chemical properties.

Analysis of bacterial cellulose production yield:

In this study, two-step transfer strategy yielded 8.8 g of BC with 68% of moisture content, which is notably higher than the yield reported by Son et al. (2001) [6] under higher shaking frequencies, thereby demonstrating the effectiveness of moderate agitation combined with pellicle transfer. The result indicates that even without replacing the standard HS medium with agro-waste substrates, productivity can be improved significantly through simple operational modifications, reducing energy input and improving efficiency. Compared to previous studies where yields ranged between 2–3 g/L in static HS cultures (Hestrin et al., 1954; Nguyen et al., 2008; Mikkelsen et al., 2009) [7, 26,10]. The present outcome suggests that relatively low-frequency agitation followed by inoculum transfer represents a promising, cost-effective, and scalable strategy for green synthesis of bacterial cellulose. By integrating both traditional HS medium-based methods and novel operational improvements, this study contributes to ongoing efforts aimed at making bacterial cellulose production more economically and environmentally sustainable [27-31].

FTIR analysis:

Fourier Transform Infrared Spectroscopy (FTIR) is one of the most widely used techniques to characterize the chemical structure and purity of bacterial cellulose (BC). The FTIR spectrum of the BC sample (Fig. 2) showed characteristic absorption peaks corresponding to functional groups typically associated with cellulose. The broad absorption band observed at 3278 cm^{-1} corresponds to the O–H stretching vibrations of intermolecular and intramolecular hydrogen bonds, confirming the extensive hydrogen-bonded network that stabilizes cellulose chains. This band is an important indicator of the hydrophilic nature of BC and its water retention ability, which aligns with its high-water holding capacity measured experimentally [32].

A peak at 2925 cm^{-1} corresponds to the C–H stretching vibrations of aliphatic groups, reflecting the polysaccharide backbone structure. The peaks at 1640 cm^{-1} and 1543 cm^{-1} are associated with absorbed water molecules due to the hygroscopic nature of BC. These peaks confirm the presence of bound water, which is typical for bacterial cellulose samples, as water molecules are retained within the nanofibrillar network. The band at 1377 cm^{-1} corresponds to C–H bending vibrations, while the peak near 1319 cm^{-1} indicates O–H in-plane bending vibrations. A strong absorption at 1233 cm^{-1} is related to C–O–C asymmetric stretching vibrations of β-1,4-glycosidic linkages, which are the fundamental bonds linking glucose monomers in cellulose chains. The distinct peak at 1028 cm^{-1} corresponds to C–O stretching vibrations in primary alcohols and β-glycosidic linkages, further confirming the polysaccharide structure. Finally, the band at 551 cm^{-1} indicates skeletal vibrations of the glucose ring, characteristic of cellulose (Pandey et al 2024a; Pandey et al 2025a).

Overall, the FTIR spectrum confirms the purity of bacterial cellulose, with no detectable peaks corresponding to proteins, lipids, or other microbial residues. This validates the effectiveness of the alkaline purification and

washing steps used in the experimental protocol. The obtained results are consistent with previously reported FTIR profiles of bacterial cellulose, which also highlight O–H, C–H, and β-glycosidic linkages as signature functional groups (Pandey et al., 2024b; Pandey et al., 2025b).

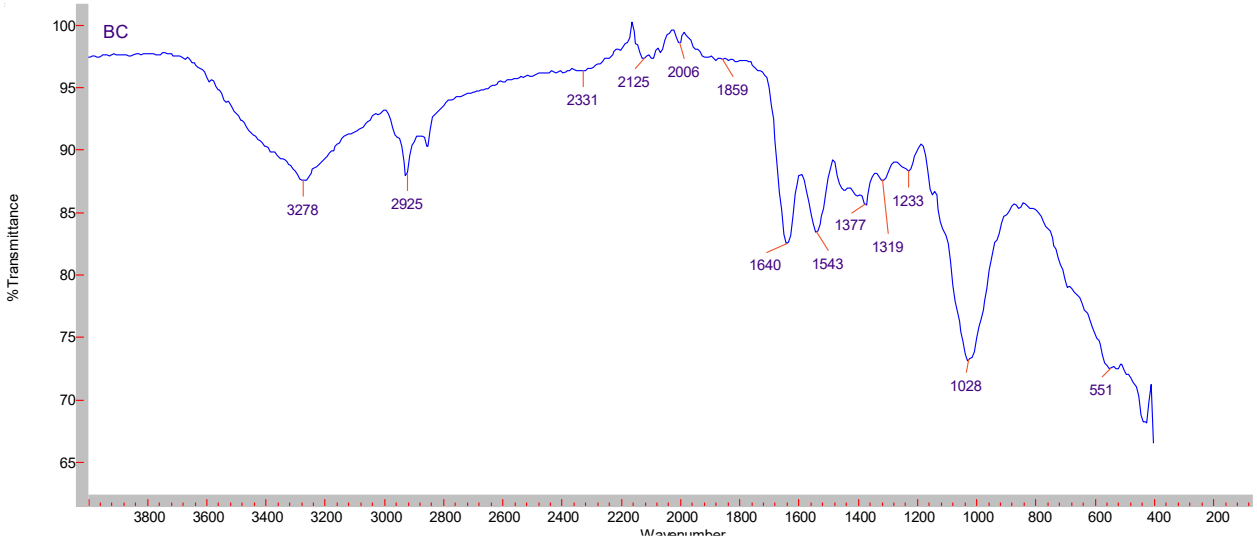

Fig.2. FTIR Graph of Bacterial cellulose.

XRD Analysis of Bacterial Cellulose

X-ray diffraction (XRD) analysis provides information about the crystalline structure, crystallinity index, crystallite size, and d-spacing of bacterial cellulose. The diffraction pattern of BC obtained in HS medium is shown in Fig. 3. Three distinct peaks were observed at 2θ values of 10.11°, 27.42°, and 32.90°, which correspond to crystalline reflections of cellulose. The peak at 2θ = 10.11° corresponds to the (1–10) plane, while the more intense peaks at 27.42° and 32.90° are attributed to (200) and (004) planes, respectively. These peaks confirm the presence of cellulose I allomorphs, particularly both Iα and Iβ crystalline forms, which are characteristic of bacterial cellulose [27-31].

The calculated d-spacing values, derived using Bragg's law, were 8.74 Å, 3.25 Å, and 2.72 Å for the three major peaks (Table 1). These values are in good agreement with standard cellulose crystallographic data. The crystallinity index of BC was determined to be 68%, indicating a highly ordered arrangement of cellulose microfibrils. This high crystallinity contributes to the superior mechanical strength, stability, and barrier properties of bacterial cellulose compared to plant cellulose, which often has lower crystallinity due to associated hemicellulose and lignin impurities. Based on the FWHM values, the average crystallite size was determined to be approximately 2.328 nm. Such nanocrystalline domains are typical of bacterial cellulose and reflect its nanoscale fibrillar network.

The XRD analysis thus confirms that BC synthesized in HS medium possesses high crystallinity and nanoscale crystallites, features that directly correlate with its mechanical performance, dimensional stability, and resistance to enzymatic degradation. These findings are consistent with earlier studies reporting crystallinity values between 60–80% for bacterial cellulose (Pandey et al., 2024a; Pandey et al., 2025a).

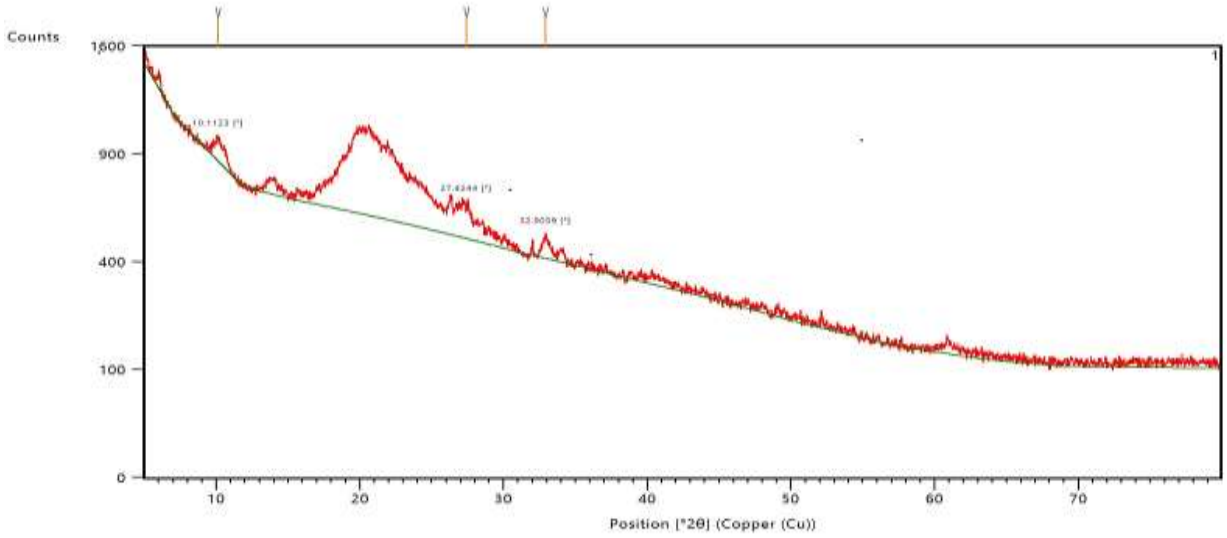

Fig.3. X-ray diffraction graph of bacterial cellulose.

Table 1: Peak list

Pos.[°2θ]	Height[cts]	FWHM Left.[°2θ]	d-spacing[Å]	Rel. Int.[%]
10.1123	129.60	0.8817	8.74757	82.18
27.4244	157.69	0.7557	3.25228	100.00
32.9059	90.42	0.5038	2.72197	57.34

Morphology of Bacterial Cellulose and Water Holding Capacity

The morphology of bacterial cellulose (BC) is strongly dependent on synthesis conditions such as dynamic and static culture environments, which directly influence the microfibril arrangement, porosity, and overall structural integrity of the material. Figure 4A presents the scanning electron microscopy (SEM) image of BC pellicles, where the fibrils appear as ribbon-like microfibrils physically interconnected through non-covalent interactions such as hydrogen bonding and van der Waals forces. The average diameter of the fibrils is approximately 4.7 µm, and these fibrils are organized into a three-dimensional network that provides mechanical stability while maintaining high flexibility. Such a hierarchical arrangement is a characteristic feature of bacterial cellulose, distinguishing it from plant-derived cellulose, which typically contains additional lignin and hemicellulose that interrupt the fibrillar alignment [27-30].

In contrast, Figure 4B illustrates the SEM micrograph of bacterial cellulose membranes, which display a highly connected porous microfibrillar network. The membrane structure exhibits regular pores and a continuous, interconnected porous framework uniformly distributed across the cross-sectional area at the micrometer scale. This interconnected porosity is essential for applications requiring fluid absorption, molecular diffusion, and nutrient transport. The membrane morphology ensures not only mechanical robustness but also facilitates the retention and exchange of water molecules, making bacterial cellulose an excellent candidate for biomedical and environmental applications (Pandey et al 2024b: Pandey et al 2025b).

The water holding capacity (WHC) of bacterial cellulose is primarily attributed to its nanoporous three-dimensional fibril network. The ribbon-like microfibrils form an extensive surface area with numerous hydroxyl groups capable of forming hydrogen bonds with water molecules. This allows BC to retain a significant amount of water relative to its dry weight, often exceeding several hundred percent. In addition, the uniform pore distribution and interconnected channels enhance the capillary action and enable rapid absorption and long-term retention of water within the structure. As a result, bacterial cellulose exhibits superior WHC compared to plant cellulose, which is hindered by the presence of hydrophobic components like lignin and waxes.

Furthermore, the synthesis conditions directly influence WHC. Under static conditions, pellicles exhibit dense but highly ordered microfibrils, leading to moderate WHC, while under dynamic conditions, the fibrils are more loosely arranged, resulting in greater porosity and enhanced WHC. This dual morphological control offers versatility in tailoring BC for specific applications such as wound dressings, food packaging, drug delivery, and water purification, where both mechanical integrity and fluid management are crucial. In summary, bacterial cellulose demonstrates a unique ribbon-like microfibril morphology with a three-dimensional, interconnected porous network that governs its exceptional water holding capacity. The combination of nanoscale fibrillar arrangement, high surface area, and hydrophilic hydroxyl functionality enables BC to function as a superior biopolymer compared to conventional cellulosic materials, making it highly valuable for advanced industrial and biomedical applications (Pandey et al 2024a: Pandey et al 2025a).

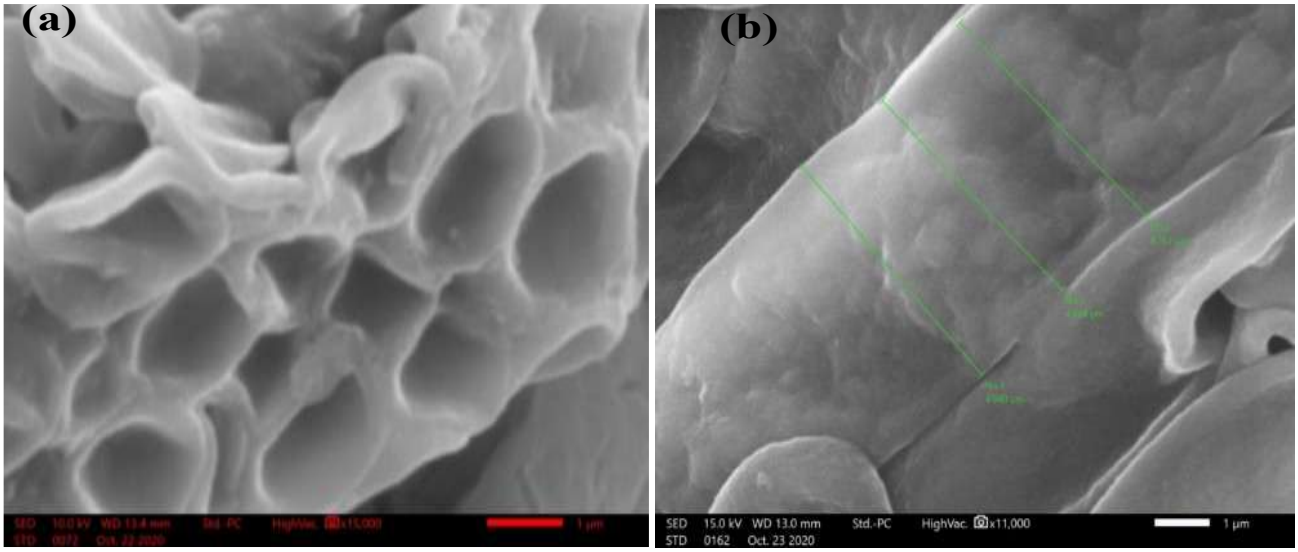

Fig.4. a is SEM Image of Bacterial cellulose pellicle and b SEM Image of Bacterial cellulose Membrane.

Conclusion:

The present study successfully demonstrated the controlled biosynthesis and morphological characterization of bacterial cellulose pellicles and membranes using *Acetobacter xylinum* NCIM 2526 under both dynamic and static conditions. SEM analysis confirmed the presence of ribbon-like microfibrils with three-dimensional assembly in pellicles and a highly interconnected porous network in membranes, which together enhance structural stability and functionality. The uniform pore distribution and nanoscale fibrillar arrangement were found to be key contributors to the remarkable water-holding capacity of bacterial cellulose.

In this study, the two-step transfer strategy yielded 8.8 g of BC with 68% moisture content, which is notably higher than the yield reported by Son et al. (2001) under higher shaking frequencies, thereby demonstrating the effectiveness of moderate agitation combined with pellicle transfer. The result indicates that even without replacing the standard HS medium with agro-waste substrates, productivity can be improved significantly through simple operational modifications, reducing energy input and improving efficiency. Compared to previous studies where yields ranged between 2–3 g/L in static HS cultures, the present outcome suggests that relatively low-frequency agitation followed by inoculum transfer represents a promising, cost-effective, and scalable strategy for green synthesis of bacterial cellulose.

By integrating both traditional HS medium-based methods and novel operational improvements, this study contributes to ongoing efforts aimed at making bacterial cellulose production more economically and environmentally sustainable. The findings further highlight the potential of BC as a multifunctional biomaterial suitable for biomedical scaffolds, wound dressings, filtration, and food packaging. Future work should focus on tailoring pore size, surface functionalization, and developing hybrid composites to broaden its applicability in specialized fields such as drug delivery and tissue engineering.

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