International Journal of Environmental and Biological Sciences
ISSN: 3054-3924

Vol. 1 , No 2 , April 2026

RESEARCH Open Access

Optimal culture and environmental circumstances for bacterial cellulose synthesis by *Bacillus licheniformis*

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Abstract:

Objectives: This study aimed to optimize bacterial cellulose (BC) production from *B. licheniformis* and characterize the produced films.

Methods: BC production was optimized by evaluating various nutrient media, carbon sources, nitrogen sources, pH levels, and temperatures. The produced BC films were characterized through solubility tests, swelling capacity measurements, in vitro degradation assays, Fourier Transform Infrared Spectroscopy (FTIR), Thermogravimetric Analysis (TGA), and Scanning Electron Microscopy (SEM).

Results: MHS nutrient medium yielded the highest BC production, 158 g/l. Glucose was the most efficient carbon source, 158 g/l. Optimal conditions were pH 7 and 35°C with a yield of 188 g/l. while yeast extract and peptone achieved maximum yields of 188 and 170 g/l, respectively. BC films exhibited insolubility in water and organic solvents, exceptional swelling capacity 2330%, and minimal degradation of 5-10% over 21 days. FTIR confirmed characteristic cellulose peaks (C-O-C, C-O, C-C, C-OH), while TGA demonstrated multistage thermal degradation and high thermal stability. SEM revealed a porous three-dimensional fibrous network with $CaCO_3$ granules integrated into the fiber structure.

Conclusion: BC was successfully produced from *B. licheniformis* under optimized conditions, demonstrating superior physicochemical properties and structural characteristics suitable for diverse industrial and biomedical applications.

Published by Democratic Arabic Center for Strategic, Political and Economic Studies (DAC)

Keywords: *Bacillus licheniformis,* production optimization, bacterial cellulose, agricultural wastes.

Introduction:

Cellulose is a naturally occurring polysaccharide synthesized by a wide variety of organisms, including higher plants, algae, bacteria, and certain types of fungi. In plants and algae, cellulose constitutes the main structural element of the cell wall, conferring rigidity and mechanical support that are vital for maintaining cell shape and withstanding environmental pressures [1]. The extraction of cellulose requires complex chemical procedures that may result in irreversible alterations to plant structure. Additionally, the procedure pollutes the environment [2,3]. BC, a potential polysaccharide of microbial origin, is typically made using inexpensive, easily accessible methods using natural or synthetic media made of different environmental wastes. For sustainable BC production, a variety of wastes from industry, agriculture, and food processing have been investigated [4]. BC is a natural polymer composed of β-D-glucose units linked through β-1,4-glycosidic bonds. These bonds form linear chains that are secreted extracellularly by specific microorganisms during the fermentation of glucose. As glucose is metabolized, it is converted into β-1,4-glucan chains, which undergo external crystallization to produce cellulose nanofibers. These fibers gradually assemble into a three-dimensional network with a gelatinous texture that accumulates at the surface of the liquid culture medium [5]. It has emerged as a highly promising biomaterial, gaining attention across multiple industries, such as food, pharmaceuticals, materials science, and textiles [6,7].

The properties of BC are influenced by various factors, including the type of microorganism used, cultivation conditions, growth medium composition, and post-production processing techniques. For example, the bacteria *Komagataeibacter xylinus* is widely known for its high efficiency in producing BC with superior purity, free from common plant impurities such as lignin and hemicellulose. However, yields and structural properties vary. Optimizing cultivation variables, pH, aeration, temperature, and nutrient availability, can significantly improve the quantity and quality of BC produced [8,9]. The high cost of the components of synthetic media significantly limits the industrial-scale production of BC, with approximately 50–65% of the production cost attributed to the components of the culture medium [10]. This makes it economically unviable. Conversely, nonspecific media, like beet molasses, pineapple peel, coconut water, fruit juices, agricultural waste, and industrial byproducts with undefined chemical compositions, are used in BC biosynthesis and are characterized by their unknown composition [11,12]. These alternative substrates not only reduce production costs but also contribute to sustainable waste management by valorizing agricultural and industrial waste. However, the use of non-specific media presents challenges, including variations in BC yield and quality from batch to batch, necessitating optimization of fermentation conditions for each substrate type. Despite these challenges, exploring low-cost media remains a crucial research focus for making BC production commercially viable [13]. The present study aimed to optimize and characterized the production of BC by *B. licheniformis* bacteria under various cultural and environmental conditions.

MATERIALS AND METHODS

Preparation of bacterial isolate

Environmental isolate of *B. licheniformis* from oil reservoirs were used in this study to produce BC. The strain was obtained from the Laboratory of Applied Microbiology, Department of Biology, College of Science, University of Basrah. In order to assess BC production, the isolate was activated on nutrient agar medium. One or two bacterial growth discs were added to 50 ml of nutrient broth in a 100 ml flask, and the mixture was shaken at 35°C for 24 hours to activate the bacteria and create the inoculum of isolates. In every trial that followed, 10% (v/v) of the activated bacteria was used as the inoculum.

Optimizing BC production under various cultural conditions

Various media and cultural conditions: Several culture media were used to determine the optimal medium for production. Hestrin and Schramm (HS) medium [14]. Modified Hestrin and Schramm (MHS) medium [15]. Mineral estimated medium (MSM) [16]. Glucose acetic acid medium (GAM) [17]. Glycerol based medium (GBM) [18]. All media were inoculated with 10% of bacterial activated culture and incubated at 35°C for 14 days in static and agitation conditions. The optimal medium and cultural conditions were determined and then employed in further experiments.

Various Carbon sources: Different natural and artificial carbon sources (starch, sucrose, fructose, date extract, beetroot juice, date juice, dried apricot juice, raisin juice, strawberry juice, whey, rice water) were used to determine the optimal source for BC production. Each natural carbon source was added at a volume of 50 ml to 50 ml of MHS medium only date extract was added at a concentration of 5% (v/v) to 100 ml of MHS medium. Artificial carbon sources were also supplemented at the same concentration 5%[19]. The optimal carbon source was subsequently determined and used in further experiments.

Optimal pH and temperature: To determine the optimum pH conditions, three pH levels (4, 7, and 8) were evaluated. The optimal PH was determined and then employed in further experiments. The effect of temperature on production efficiency was performed at two different temperatures, 25 and 30°C. The optimal temperature was determined and then employed in further experiments.

Various nitrogen sources: Multiple nitrogen sources including (peptone, urea, KNO_3, $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, and $NaNO_2$) were evaluated. Each nitrogen source was also added to the culture medium at 0.5% separately. The optimal nitrogen source was determined and then employed in further experiments.

Production, extraction, and purification of BC

The beast culture media, carbon and nitrogen sources, and environmental factors were used for the production of BC. After being isolated of BC in the production medium, the surface layer underwent a number of purification procedures. To get rid of any last traces of the culture media, it was first repeatedly rinsed with distilled water. The layer was then exposed to 1% (w/v) NaOH for ten min. And repeatedly cleaned with distilled water to guarantee the total removal of any remaining alkaline residues. The layer was then dried at room temperature, autoclaved to remove any remaining bacteria and spores [20].

Characterization of BC films

Solubility test: The solubility of BC film was evaluated in various solvents, including water, ethanol, chloroform, dimethyl sulfoxide (DMSO), propanol, xylene, methanol, butanol, isopropanol, acetic acid, ammonia solution, and a mixed solvent system consisting of 7% NaOH, 12% urea, and 81% D.W.[21].

Swelling test and in vitro degradation analysis: In vitro degradation analysis of BC was conducted according to [22]. The swelling capacity of BC was assessed using a gravimetric method under ambient conditions according to [23].

Thermogravimetric analysis (TGA): Thermogravimetric analysis was conducted using a SDT Q600 V20.9 thermogravimetric analyzer (Model DSC-TGA) at the Taban Lab in Tehran, Iran. Sample with an average dry weight of 2.655 ± 1 mg were analyzed under a continuous argon purge (40 ml/min flow rate). The temperature program was executed at a constant heating rate of 20°C/min, scanning from 24°C to 1008.61°C.

Fourier transform-infrared spectroscopy (FT-IR): FTIR spectroscopy was conducted using an FTIR spectrometer (FTIR alpha ll /Germany, Bruker) within a wave range of 400-3900 cm^{-1}.

Scanning electron microscopy (SEM): The surface morphology of the BC film was examined using a MIRA3 scanning electron microscope at Shiraz University, Iran. The sample was coated with a thin layer of gold. SEM analysis was performed at an accelerating voltage of 20 kV, and micrographs were acquired at different magnifications.

Statistical analysis

Every experiment was carried out in triplicate as separate biological replicates, including assays for BC production. Statistical significance was determined using chi-square test and ANOVA.nalyzed with SPSS version 26 (2019).

Results and Discussion

Screening of optimum cultural conditions for the production of BC

Different media and cultural conditions:

To determine the most suitable culture medium for BC production, five different media were tested under static and agitation incubation conditions. The results showed that the isolate revealed production under the static condition, while there was no production under the agitation condition. Under the static condition, the yield of BC for each medium is presented in (Figure 1). The optimum medium for BC production was MHS medium, which resulted in a significantly ($p < 0.001$) higher yield of 158 ± 1.41 g/l compared to the other tested media.

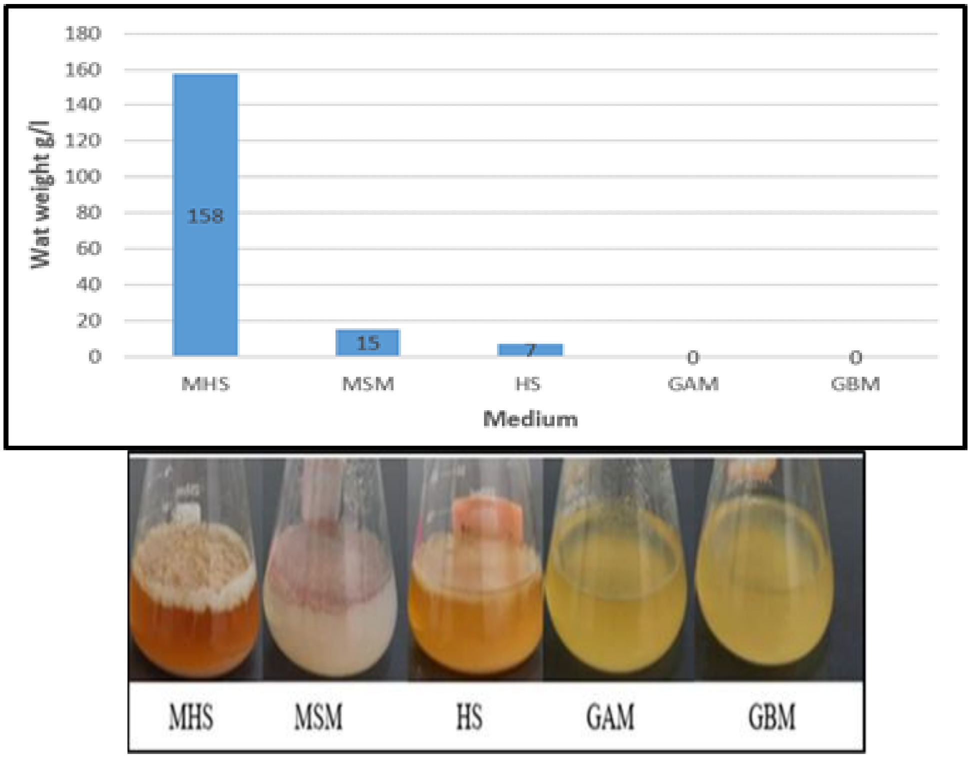


Figure 1: Optimization of BC on different culture media.

Carbon sources: To evaluate the impact of different carbon sources on BC production, a variety of natural and synthetic substrates were tested using the MHS medium. The results showed that glucose was the most optimum source with a significantly ($p < 0.001$) increased yield of 158± 1.41 g/l (Figure 2 and 3a).

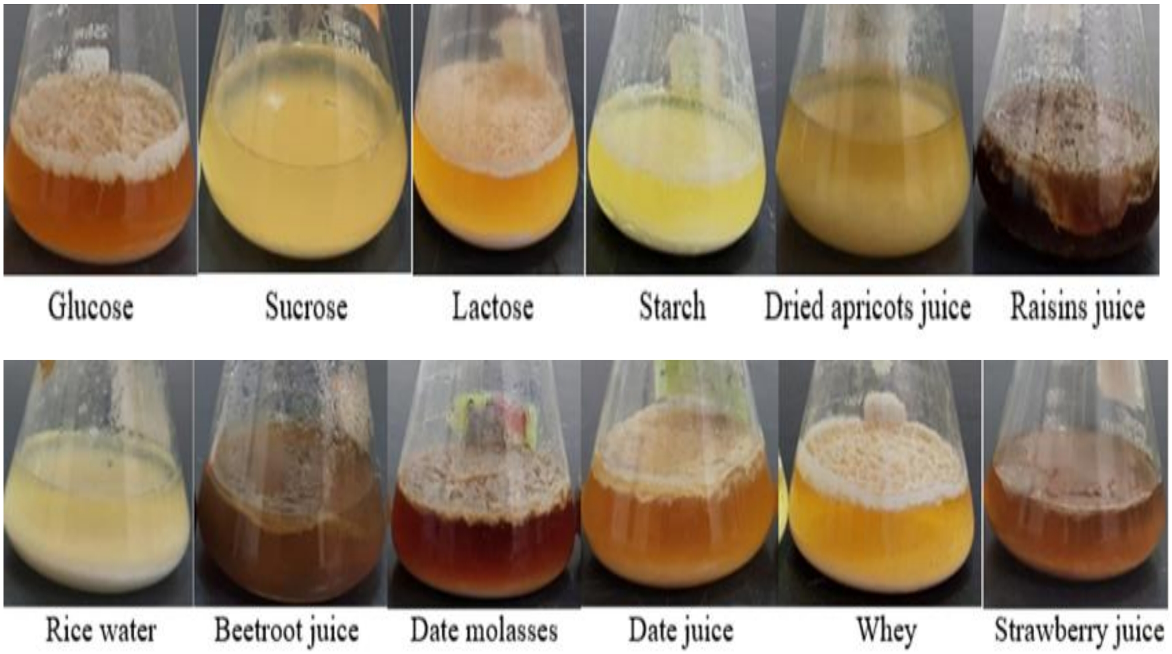
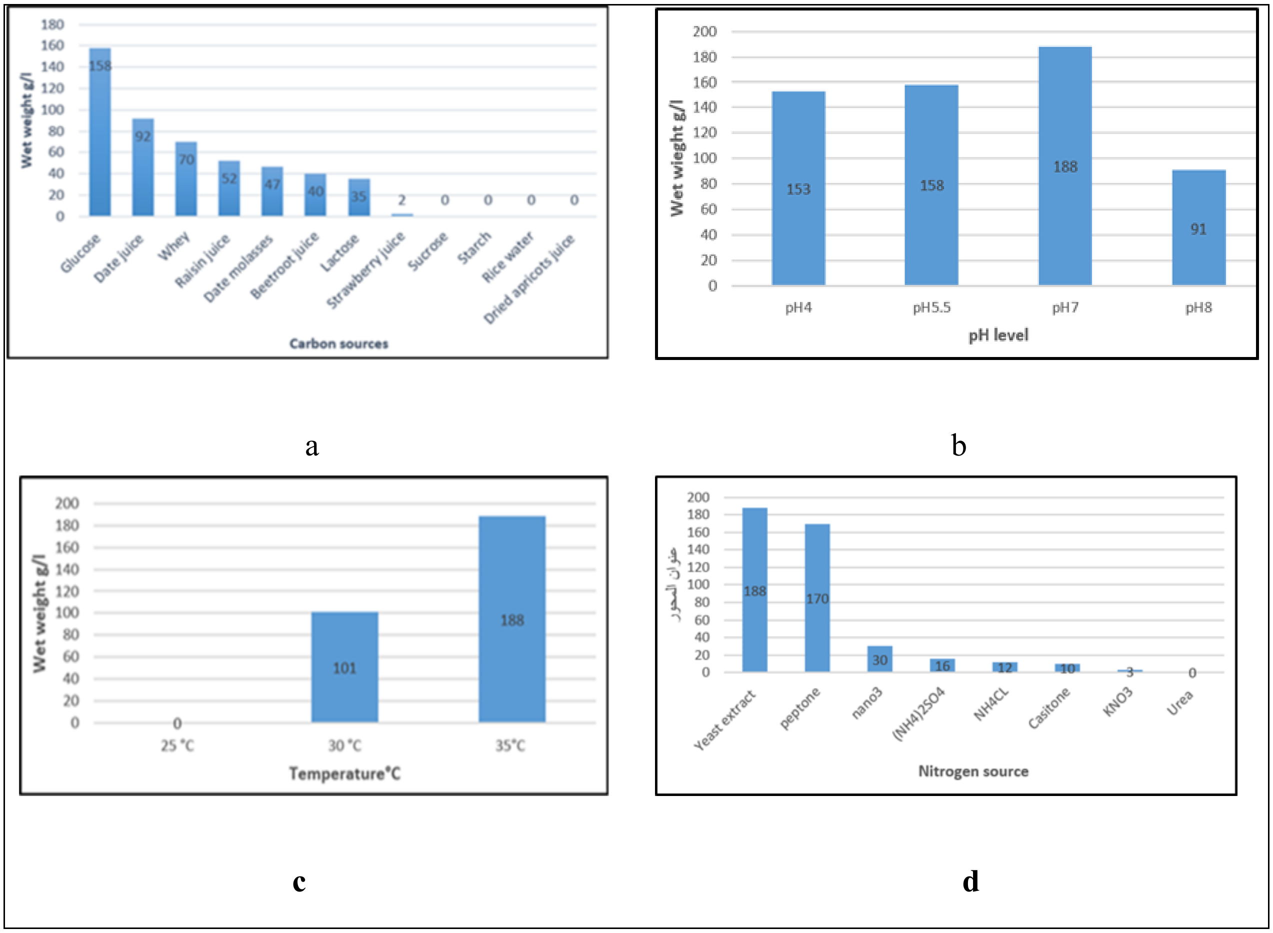


Figure 2: The effect of various carbon sources on BC production by *B. licheniformis*.

Optimum pH and temperature: To determine the optimal pH for the production of BC, three different pH values were tested. The results showed that pH 7 was the optimum condition for production, yielding 188 ± 1.41 g/L, and was significantly higher than other tested pH values ($p < 0.001$). A decrease in yield was observed at pH 8.0 and 4.0, 91± 1.41 and 153± 1.41 g/l, respectively (Figure 3b). To determine the optimal temperature, different temperatures were tested. The result showed that a temperature of 35°C was optimal for production, with a yield of 188± 1.41 g/l, ($p < 0.001$). No yield was observed at 25°C (Figure 3c).

Nitrogen sources: The influence of different nitrogen sources showed that the yeast extract and peptone were the perfect sources for the production of BC, with significantly ($p < 0.001$) yields 188± 1.41 and 170± 1.41 g/l, respectively (Figure 3d).



a

b

c

d

Figure (3): The yields of BC produced. a. Different carbon sources. b. Different nitrogen sources. c. Different pH levels. d. Different temperature.

Extraction and purification of BC:

The best cultural conditions and environmental factors that give efficient production were used for the production of BC. The BC surface layer was successfully isolated from the production medium. The film was a brown leather-like color, a highly flexible film with a spongy and gelatinous mat-like structure. The thickness of the film was about 2 mm (Figure 4).

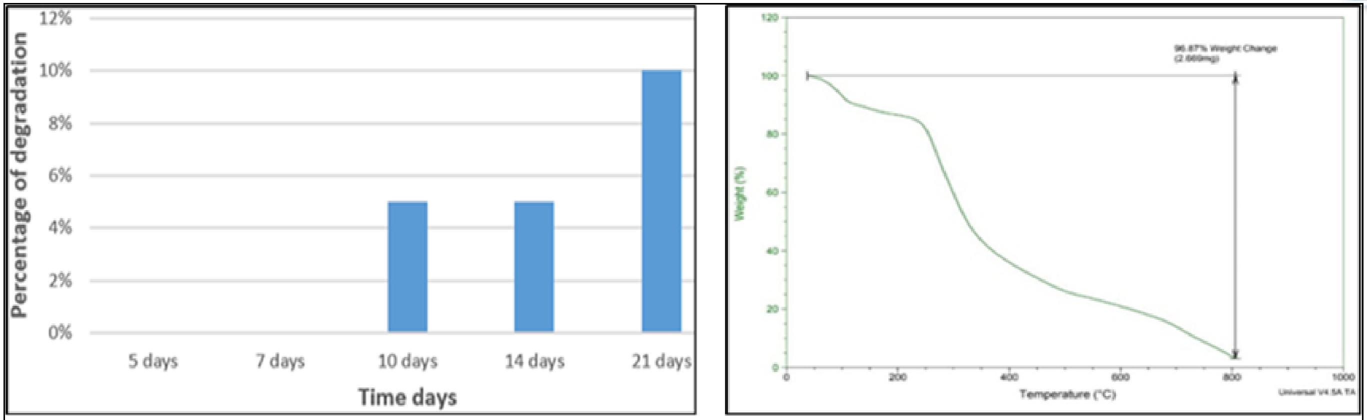
Figure 4: Production of BC by *B. licheniformis*: a. Before purification. b. After purification. c. after drying.

Characteristics of BC films: solubility, swelling test, and in vitro degradation:

The solubility test of BC was performed in different organic solvents and water. The results showed that the BC was insoluble in all solvents except a unique solvent combination (7% NaOH,12% urea, and 81% water). The results of the swelling test showed that the BC film has a high swelling capacity of 2330%, while degradation of the BC film was achieved over 21 days. The results revealed a moderate and regulated degradation rate, ranging from 5% after 10 days to 10% after 21 days for BC (Figure 5a).

Thermogravimetric analysis (TGA):

The results of BC thermal analysis revealed expected thermal. As illustrated in the (Figure 5b). The BC film exhibited an initial weight loss of 13.5% between 0 and 200°C, primarily due to moisture evaporation from the fibrous matrix. The greatest decomposition occurred between 200 and 400°C, representing a loss of 64%. At 500°C, the total weight loss reached 74%. A 50% weight loss was recorded at 324°C, demonstrating moderate thermal stability. At 806°C, only 3.1% of the original mass remained, confirming that the material had undergone almost complete thermal decomposition.



a

b

Figure 5: BC characterization: a. In vitro degradation of BC film during 21 days. b. The TGA of BC.

FT-IR analysis of the BC: The FTIR spectroscopy was used to assess the chemical compounds and functional groups of the BC film. The results were illustrated in the (Table1) and (Figure 6).

Table (1). Main functional groups of BC identified by FTIR.

Wave number	Stretching	Expected compounds	Appearance
3287.80	O-H stretching (broad)	Broad OH-ring typical of cellulose—indicating internal and external hydrogen bonds.	Strong broad
[2925.01 & 2865.07	C - H (CH3) and (CH2)	Vibrations of –CH / –CH₂ in sugar rings and cellulose chains.	Medium
1632.24	C=O/H-O-H	Carboxylate group/adsorbed water	Medium
1596.11	COO^- asymmetric	organic/aromatic compounds	Medium
1539.82	N-H	amide/ peptide (contaminants)	Medium
1390.71	C-H	Aldehyde	Medium
1026.30	C-O-C/ C-O	Glycosidic linkages/Strong evidence for the glycosidic structure of cellulose	Strong
870.20-781.45	C-H	Glycosidic linkage	Strong
663.51 & 642.64	C-Br /C-Cl	halo compound	Strong

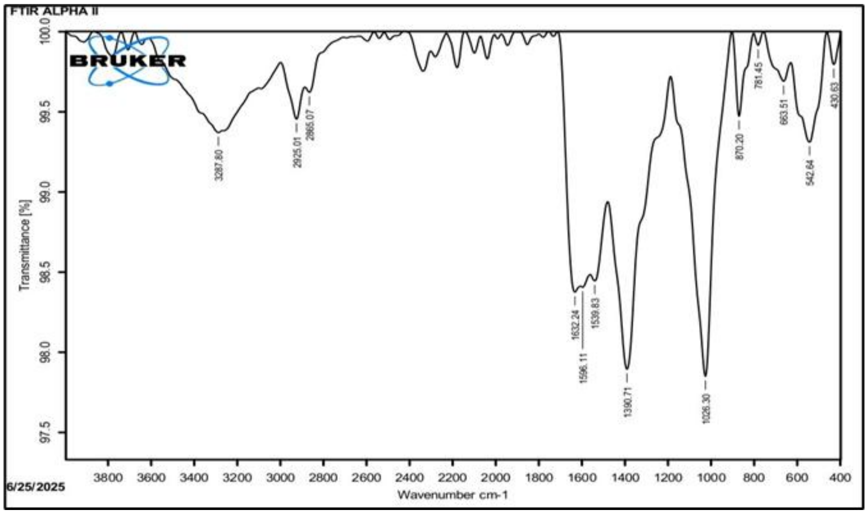


Figure 6: FT-IR analysis of the BC film.

BC film morphological characteristics as determined by SEM:

The SEM was used to assess the thorough morphological characterization of dried BC films. The BC structure is depicted in (Figures 7 a, b and c), with low- and high-magnification images 111x,160x, and 457x, respectively. The images display important visual information on the morphology of the BC. It clearly reveals the homogeneous tridimensional (3D) porous network structure of BC microfibers; the fibers were thick and large in size. The BC surface is irregular and rough, related to the fibrous structure of cellulose, the clear and thick intertwined fibers (microfibrous cellulose), with many pores developed between them, reflecting the porous nature of the polymer. There were visible granules precipitated within BC microfibers produced by the MHS medium.

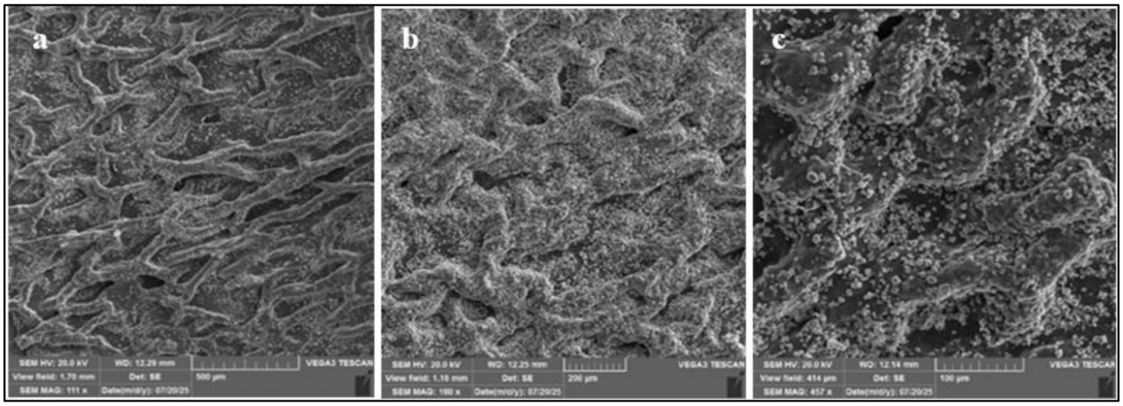


Figure 7: The SEM images of BC. a. with 111x. b. with 160x. c. with 457x.

Screening of optimum cultural conditions for the production of BC

The optimum medium for the production of BC was MHS medium with the significantly ($p < 0.001$) highest yield of 158± 1.41g/l. Many earlier studies showed the same result [15] .[24]reported that the medium composition, including carbon and nitrogen sources, mineral sources, has a major impact on BC production. MHS medium, despite having few and basic components, achieves a high yield due to its abundance of carbon sources, $CaCO_3$, and glucose [25]. The carbon source resulted in a significantly ($p < 0.001$) higher yield of BC compared to other sources 158± 1.41g/l (Figure 2and 3a). These results were in come with previous studies [26,27]. supplemented with glucose, because it is thought to be the main source of BC production. Since it is a precursor for cellulose synthesis, all compounds that can be transformed into glucose are capable of forming BC [28]. [29] showed that glucose was a perfect precursor for the *G. xylinus* to employ in the polymerization of cellulose.

In terms of pH and temperature, the best parameters for production were found to be pH 7 and 35 °C, which produced a maximum of 188 ± 1.41 g/L with statistically significant differences compared to other examined circumstances ($p < 0.001$), (Figure 3c and d). These findings were consistent with previous studies [30,31,32]. A decrease in yield was observed at pH 8.0, and 4.0, highly acidic and alkaline environments were associated with significantly reduced BC production, likely due to impaired enzymatic activity and reduced bacterial viability, as observed by [33]. Also, the optimal temperature production was in line with earlier research [34]. By directly influencing enzyme activity and metabolic rates, temperature plays a crucial role in controlling the physiological processes of microbes and aids in the development of their adaptive survival strategies [31]. [3] refer that favored temperatures for the production by *B. licheniformis* were 50°C and 37°C.

The influence of different nitrogen sources revealed that yeast extract and peptone were shown to be the best nitrogen sources for BC synthesis, with yields of 188 ± 1.41 and 170 ± 1.41 g/l, respectively, which were significantly greater than those of other examined sources, (Figure 3b). These results were in come with [27,35]. This superiority is ascribed to the yeast extract high quantities of vital vitamins and particular growth hormones, as well as the amino acids and peptides it contains, which directly assist bacterial metabolic activities [27].

Extraction and purification of BC

The BC surface layer was successfully isolated from the production medium. Sequential rinsing with distilled water effectively reduced residual medium components, which indicates efficient preliminary cleaning. The medium was treated with 1% (w/v) NaOH for 10 min. NaOH works by breaking down proteins and cellular debris, dissolving amphiphilic compounds such as fatty acids and lipopolysaccharides (LPS), and reducing microbial load [9]. The treatment visibly enhanced the clarity of the BC film, reflecting the removal of cellular debris and associated impurities. Final washes with distilled water yielded a neutral pH, confirming the complete elimination of alkaline residues (Figure 4). Despite the effectiveness of this treatment, some spores may remain resistant, so a final autoclave sterilization was carried out to ensure the complete elimination of microorganisms while preserving the properties of bacterial cellulose [9].

Characteristics of BC films

The characterization of BC film includes solubility, swelling, and in vitro degradation and TGA were performed. The results manifest that the BC was insoluble in all solvents. This result was in agreement with previous studies [19,21]. Its insolubility is due to the strong hydrogen bonds holding. The BC molecules, together with their extremely polar nature, are responsible for this behavior[19]. BC film has a high swelling capacity of 2330%. This was in line with other investigations [36,37]. The high swelling capacity, due to its hydrophilic nature and porous structure, enables it to absorb a large amount of liquid [38]. Hydrogen bonds are created when water readily penetrates cellulose and breaks the intermolecular van der Waals interactions [39]. The results of in vitro degradation showed a moderate and regulated degradation rate. The water molecules attach to the BNC fibers, causing this degradation, which dissociates the partial C-O-C bonds and weakens the intra- and inter-chain connections [40].

The results of BC thermal analysis (TGA) showed expected thermal behavior consistent with published scientific literature. The initial weight loss of 13.5% in the range 0-200°C is primarily attributed to the evaporation of moisture trapped within the BC fibrous matrix. This behavior is consistent with previous studies [41,42], which confirmed that the mass loss of BC during the first decomposition stage (≤ 200°C) is due to water evaporation. The major decomposition occurred between 200-400°C, with a weight loss of 64%, representing the dissociation of cellulose chains and the breaking of glycosidic bonds[43]. [44] reported a weight loss of approximately 83% at 292°C, (Figure 5).

FT-IR analysis of the BC

The BC film infrared spectrum revealed absorption peaks characteristic of this particular polymer. The peak of absorption in the one can be identified at 3287.8 cm⁻¹, which corresponds to the stretching vibrations of hydroxyl groups in cellulose, engaged in hydrogen-bond formation. The broader peak for BC indicated stronger OH bonding [45]. The intense band at 2925 cm⁻¹ and 2865 cm⁻¹ corresponds to the stretching vibration group of CH_2 and CH. The band located at 1632.24 cm⁻¹ has been attributed to the COOH group, which represents glucose carbonyl [46] or H-O-H stretching of adsorbed water [47]. Peaks at 1026.3 cm⁻¹ were formed by the C-O-C group and hydroxyl C-O-H functional groups of the carbohydrate ring, it's a distinct region of glycosidic linkages Β-1,4 and saccharide chains, which is strong evidence of cellulose structure. This was consistent with earlier research [48]. Furthermore, maxima for the C=O bending vibrations were found at 1596 cm⁻¹ and 1539 cm⁻¹ [27]. The band 1539 cm⁻¹ corresponding to N-H or C-N stretching indicated protein and other impurities on raw BC may be contaminants from culture media [49]. Amine functional groups may be incorporated into the BC as a result of the presence of nitrogen sources in the culture medium used to produce raw BC.

BC film morphological characteristics as determined by SEM

SEM images of BC film, showed a homogeneous surface with a three-dimensional, interconnected, porous, fibrous network structure. a characteristic pattern of cellulose structure. The porous structure provides a large surface area and multiple binding sites, promoting functional applications or the incorporation of other compounds, consistent with the usual morphological description of BC [50]. From the appearance of the fibers, it was thick and large in size. This is consistent with [31,51], showed that the largest bundles of BC were formed on glucose medium compared to other carbon sources.

The higher magnification image (Figure 7c), showed a randomly distributed fiber network that forms a highly porous matrix; this structure was helpful to absorb a large amount of water and materials. The porous structure aligned with the BC films, highly swelling 2330%, It is commonly known that BC porosity and the tunnels it forms create an intumescent fibrous network that can hold twice as much water as it weighs [52]. The images of SEM showed the presence of $CaCO_3$ granules in the BC films produced with the MHS medium. Some granules in the culture medium may be bound to the BC structure. [53]note the presence of starch granules on the surface of the BC produced with hydrolyzed starch. $CaCO_3$ is slightly soluble in water, so it precipitates in the culture medium, enabling it to adhere to the BC fibers despite the washing and purification processes. The presence of these particles may give the polymer important properties that may be useful in many applications. This was indicated by [54]. Fabricated a BNC-$CaCO_3$ hybrid biocomposite. The implants made of BNC-$CaCO_3$ bionanocomposite would be a good option for tissue engineering of artificial skin, blood vessels, bones, cartilage, wound healing, and medication delivery [55].

Conclusion

The study demonstrated that *B. licheniformis* isolated from Iraqi oil reservoirs was quite effective in BC production highlighting the importance of harsh environments as a new and promising microbial resource. MHS medium, glucose as a carbon source, and yeast extract as a nitrogen source, along with a neutral pH 7 and a temperature of 35°C, provided the optimal production conditions. The resulting films exhibited distinctive physical properties such as high swelling capacity, significant water retention, thermal stability, and a moderate degradation rate, confirming their suitability for regulated biological and medical applications.

Author contributions: All authors have reviewed and approved the final version of the manuscript for publication.

Funding: This research did not receive any external funding.

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