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Bacterial Cellulose Loaded with Amoxicillin/ Flucloxacillin: Innovate Tool for Antibacterial Applications

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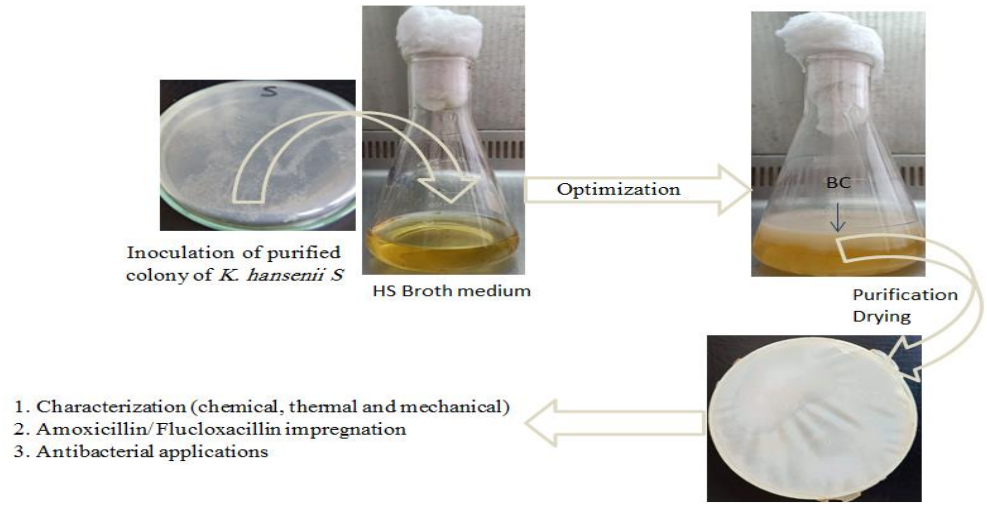
HIGHLIGHTS

1- Bacterial cellulose (BC) is a biopolymer but, the problem that limited the industrial production of BC is its low yield.

2- Amoxicillin/Flucloxacillin-impregnated BC nanofibers exhibited a high antibacterial activity against both Gram-positive and Gram-negative bacteria.

3- Chemical, and mechanical properties of BC produced by Komagataeibacter hansenii S indicate its availability to be applied in food and medical applications.

GRAPHICAL ABSTRACT



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ABSTRACT

It is urgently needed to develop an ideal biopolymer with unique physicochemical and mechanical features that match its uses in different applications such as food packaging, wound dressing, and fuel cell membranes. Bacterial cellulose (BC) is a promissory biopolymer owing to its fascinating physicochemical features such as purity, biodegradability, biocompatibility, nanofiber structure, high crystallinity, water holding capacity and mechanical stability. One of the problems that limited the industrial production of BC is its low yield. A high-producing strain of this highly physicochemical biopolymer features is needed to benefit from its enormous uses in the food and medical applications. Bacterial cellulose (BC) synthesizing *Komagataeibacter hansenii* S strain was isolated from strawberry, and the 16S rDNA sequence homologies reached 99% with *Komagataeibacter hansenii* strain LMG 1527 so that the isolate can be identified as *Komagataeibacter hansenii* S. The isolated strain was selected owing to its high production yield of (5.4±0.6 g/l, dry weight) under optimized culturing conditions. The morphological and physicochemical features of the produced BC were studied by SEM, FTIR, XRD, TG, and mechanical tests. FESEM showed the three-dimensional porous nanofiber structure. FTIR and XRD confirmed the structure of BC. TG revealed the thermal stability of produced BC with good mechanical properties. Amoxicillin/ Flucloxacillin impregnated BC nanofibers exhibited high antibacterial potency against both Gram-positive and Gram-negative bacteria. Physicochemical and mechanical features of BC produced by *Komagataeibacter hansenii* S indicate its availability to be explored in food packaging, wound dressing, and fuel cell membranes.

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1. INTRODUCTION

In many natural habitats, many bacteria produce extracellular polysaccharides, some of them mostly gram-negative bacteria can produce extracellular gel substances in a liquid sugar matrix called Bacterial cellulose (BC) [1, 2].

The BC biosynthesis was firstly observed by the ancient Chineseand they recovered it from kombucha tea fermented beverage [3]. The BC-producing strains are obligatory aerobic gram-negative bacteria such as *Gluconacetobacter* (especially *Acetobacter* and recently, *Komagataeibacter*), *Aerobacter*, *Azotobacter*, *Achromobacter*, *Rhizobium*, and *Agrobacterium* [4]. It was reported that members of the *Gluconacetobacter* genus are the most highly BC producers among other strains [1, 5]. BC has been found to have a chemical formula that matches plant cellulose, but it has many advantages over plant cellulose including high purity, degree of polymerization, crystallinity, water holding capacity, surface area, thermal stability, and mechanical properties [6]. BC microfibrils are approximately one hundred times thinner than the fibrils of vegetal cellulose [7]. These amazing physicochemical features have aroused worldwide attention from researchers and industrial sectors. In food sectors, BC was approved as a food ingredient by the US Food and Drug Administration (FDA) [8]. BC has been used as a fat replacer and meat analog [9]., stabilizer Pickering emulsions [10]., and food packaging applications [11]. Recently, BC/AgNP composite was obtained by using BC as a reducing agent for food packaging, and wound dressing applications [5]. The main problem facing BC production is its low production yield, therefore, to increase the production of BC from *Komagataeibacter* strains, various favorable conditions such as incubation temperature of cultures and pH of the used media as well as the composition of cultivation media have been tried [12, 13, 14].

In this study, strawberries were used to isolate the BC-producing strain. The isolate was identified through the study of physiological, biochemical properties and analysis of the 16S rRNA sequence. In addition, the effect of different media composition, incubation period, temperature, pH, glucose concentration, ethanol concentration, and gamma radiation doses on BC production were also investigated. The produced BC was characterized by scanning electron microscopy (SEM), X-ray Diffraction (XRD), Fourier Transform Infrared Spectra (FT-IR), Thermogravimetric Analysis (TGA), and Tensile test.

2. MATERIALS AND METHODS

2.1. Isolation of BC-producing strain

Rotten strawberry samples were collected from fruit markets at Great Cairo and used for the isolation of bacteria. Twenty-Five Grams of the strawberry sample were transferred into a 250 ml conical flask containing 50 ml of modified Hestrin and Schramm (HS) broth medium (% w/v): 2% glucose, 0.5% yeast extract, 0.5% peptone, 1.5% ethanol, 0.27% Na_2HPO_4, and 0.15% citric acid [15]. The flasks were left to be statically incubated at 30ºC for 7 days. The formation of a white pellicle covering the surface of the media was a preliminary indication of BC production. Only cultures with white pellicle (non-dissolved) were selected and purified after several streaks on HS agar plates and then used for BC production. The produced BC pellicles were purified and dried according to a previously reported procedure [5].

2.2. Identification of BC-producing bacterial isolates

The identification of the isolated BC-producing bacteria was based on its morphological, and biochemical characteristics, as well as the 16S rRNA gene sequence. Gram staining and cell shape were applied for fresh cultures of all screened isolates. The physiological and biochemical characteristics of the isolates were tested according to Berge's Manual of Systematic Bacteriology [16]. The genomic DNA was extracted for PCR on 16S rDNA with the forward primer: (5'- AGA GTT TGATCC TGG CTC AG -3'), and the reverse primer: (5'- GGT TAC CTT GTT ACG ACT T -3'). PCR products were purified and sequenced by GATC Company (Germany).

2.3. Effect of different factors on BC production

Different incubation temperatures (15, 20, 25, 30, 35 and 40 °C), time (1-10 days) and inoculum age (24, 48 and 72h) were applied. Glucose (2%), in the modified HS medium, was replaced with other carbon sources (sucrose, maltose, fructose, galactose, mannitol, arabinose, melibiose, and trehalose). Similarly, the effect of different nitrogen sources (yeast extract, peptone, beef extract and potassium nitrate) were evaluated by keeping other factors constant except the yeast extract and peptone (0.5%). Different concentrations of glucose (1-5%) and ethanol (1- 2.5%, V/V) were examined. The effect of gamma-ray on BC production was examined; 10 ml of 48h fresh cells grown on optimized medium were exposed to different gamma radiation doses (0.2, 0.4, 0.6, and 1kGy). 1 ml of irradiated cell suspension of

each tube was cultivated in a 250 ml-conical flask containing 50 ml of optimized medium. The flasks were then incubated at the previously optimized conditions; BC was harvested, washed, dried, and weighted as previously described. The irradiation process was carried out with Co^{60} gamma radiation source. The dose rate of this radiation source was 2.08kGy/h at the time of the experiment.

2.4. Characterization of BC

Morphological analysis of the surface of the dried BC film was performed by a JEOL (JSM – 5500 LV, Japan) scanning electron microscopy (SEM). Fourier transform infrared (FTIR) spectrum was performed on ATI Mattson Genesis series (England) at a frequency range of 4000−400 cm−1. The XRD pattern of the dried BC was recorded on a Shimadzu XRD-6000 (Japan). Thermogravimetric (TG) and derivative thermogravimetric (DTG) of the BC pellicle samples were evaluated using Thermogravimetric Analyzer (TGA-50 Shimadzu, Japan). BC pellicle samples of 1.1 mg were weighed in Ni cells and scanned from 50 to 600 at a flow rate of 20 ml/min in the presence of nitrogen. The moisture content was calculated as the percentage of weight loss. Tensile strength (MPa) and elongation were measured from stress-strain curves using Tension – meter (Mecmesin, Multites 10 – I, Germany), the tension speed was 15 mm and samples were in a dumbbell shape (5 cm long and 1 mm width).

2.5. Antibacterial potency of Amoxycillin / Flucloxacillin impregnated BC

One capsule of Flumox (EIPICO Company, containing Amoxycillin (250 mg) and Flucloxacillin (250 mg) was completely dissolved in 5 ml sterile distilled water; different discs (6mm) of the produced BC were dried and weighed until obtaining 3 successive readings. The discs of BC were loaded with 10µl of the dissolved capsule, dried, and tested as well as, BC was tested against both gram-positive (*Staphylococcus aureus* ATCC 6538) and Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Klebsiella pneumonia* ATCC 27736 and *Aeromonas hydrophila* ATCC 7965).

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of BC producing bacteria

Twenty-five bacterial isolates were isolated from different rotted strawberry samples. They were screened for BC production on a modified HS broth medium. Only one isolate (S) had the ability to produce bio-cellulose. The bacterial cells were Gram-negative with rod shape under the microscope, while on modified HS plates the colonies were circular round, creamy whitish, and smooth. Based on the morphological results, physiological and biochemical test results of the S isolate and based on the Manual of Systematic Bacteriology [1]. The isolate was identified as *Gluconacetobacter* (Table1). The 16S rRNA gene sequence was compared with several related taxia in the NCB1 database. According to that, the bacterial isolate under investigation had a homology, similarity of 99% with *Komagataeibacter hansenii* LMG1527 (Fig.1). The BC production ability is widespread among several bacterial species, but the most BC-producer one is the species *Komagataeibacter xylinus*, also other species, such as *Komagataeibacter hansenii*, *Komagataeibacter rhaeticus*, *Komagataeibacter saccharivorans* and *Komagataeibacter pomaceti* have been reported as strong cellulose producers [5, 17]. The synthesis of BC in bacterial cells follows several steps: (i) BC is synthesized in the bacterial cell membrane through activation of glucose nucleotide as the precursor for BC synthesis, (ii) polymerization of UDP-glucose by the polymerizing enzyme (cellulose synthase) which exists in the cell membrane of BC producing bacteria, (iii) assembly of glucose chains into bundles and ribbons (iv) secretion of ribbons through the wall/membrane into the extracellular matrix [18].

Table (1): Biochemical tests of S isolate

Tests	Results
Cell shape	Rod
Arrangement of cells	Singly, in chains
Gram staining	-
Motility	-
Oxidase	-
Catalase	+
Urease	-
Indole production	-
H_2S production	-
Citrate utilization	-
Formation of brown pigment	+
Oxidation of acetate	+
Oxidation of lactate	+
Production of BC	+
D-glucose	+
Galactose	+
Manitol	+
Sucrose	+
Arabinose	+
Melibiose	+
Inositol	-
Maltose	+
Xylose	+

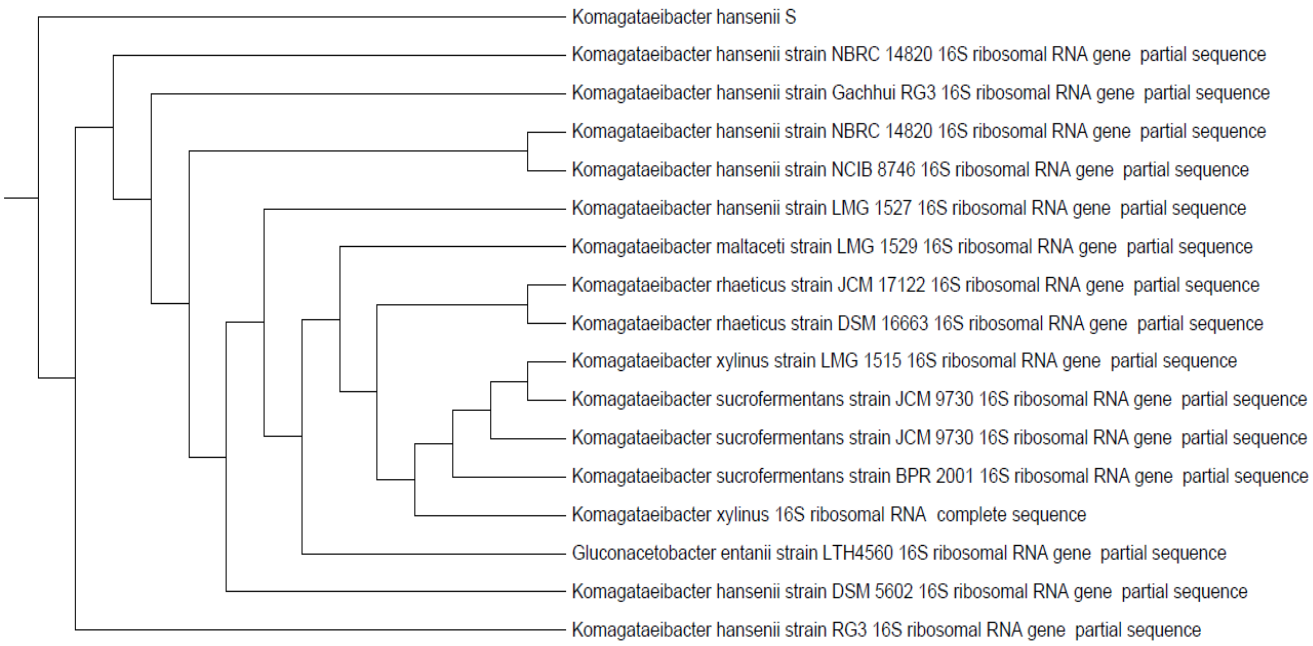

Fig. (1): Phylogenetic tree based on 16S rRNA gene sequences of *Komagataeibacter hansenii* S and the related taxa.

3.2. Optimization of environmental and nutrient parameters for BC production

The main problem facing the industrial application of BC is its low yield by fermentation cultures. To increase the production yield of BC by *Komagataeibacter hansenii* S (*K. hansenii* S), several factors were optimized (Fig. 2). Different temperatures, 15°C, 20°C, 25°C, 30°C, 35°C, and 45°C were evaluated for the maximum production of BC by *K. hansenii* N. The maximum BC production was observed at 30°C of about 0.73±0.11g/l. As the temperature decreases or increases, BC yield also gets decreasing at 20°C, 25°C, and 35°C. The temperature values of 40°C and 45°C showed no BC production. The maximum production of BC was recorded after 8 days of incubation (0.74±0.04) with no significant increase in the BC production at 9 and 10 days. The maximum BC production was obtained at pH 6.0 (1.1±0.15 g/l), followed by pH 7.0 (0.86±0.11 g/l), the pH values of 2.0 and 9.0 showed no BC production yield.

BC production was evaluated by supplementing with nine carbon sources, five nitrogen sources, different concentrations of glucose (from 1 to 5 %), different concentrations of ethanol (from 0.0 to 2.5 %), and different doses of gamma-ray (0.0-1.0 kGy). Among the used carbon sources, glucose produced a maximum BC yield (2.3±0.23 g/l), followed by mannitol (1.3±0.2 g/l) and sucrose (0.62±0.17 g/l). There was no significant difference in the yield of the produced BC from the other tested carbon sources. It was observed that increasing glucose concentrations (1-4%W/V) to the fermentation medium greatly increased the production of BC. At different concentrations of Glucose (1 to 5 %) and the maximum production of BC (5±0.4 g/l) was produced at 4% of Glucose concentration. At concentration of 5 %, the BC production yield was decreased (4.3±0.3 g/l). The results agree with the observations of Son et al. [13].and Castro et al.[17]. In most cases, glucose has priority over other carbon sources as it represents the best energy source for bacterial cells, and it can be directly used as an ideal precursor for the assembly of glucose units into bacterial cellulose [19].

A nitrogen source is also important as it can provide the BC-producing bacteria with amino acids, mineral salts, and vitamins. Among the nitrogen sources used, a combination of yeast extract (0.5%) and peptone (0.5%) gave the highest BC production yield (2.5±0.3), followed by yeast extract (0.5%) which produced a yield of 1.6±0.2 g/l. Next to yeast extract, peptone produced 1±0.15 of BC. Yeast extract and peptone are the most preferred nitrogen sources for BC-producing bacteria [16, 20]. The addition of ethanol at different concentrations from 0.5 to 2.5% (V/V) to the fermented medium led to a significant increase in the production of BC with the highest production of 5.4±0.6 g/l at 1.5% ethanol which was about 6 times more than the produced yield without ethanol. The use of ethanol for enhancing BC production has been studied by many investigators. The role of ethanol in influencing the cellulose synthase enzyme is clearly obvious. Ethanol, at optimum concentration, is known to enhance the production of adenosine triphosphate (ATP), In the BC

synthesis pathway; ATP promotes the activities of fructokinase and glucokinase, in contrast, inhibits glucokinase activity and glucose-6-phosphate (G6P) dehydrogenase in pentose phosphate pathway [21]. Besides, ethanol can degenerate spontaneous mutation into cellulose non-producing (Cel−) mutants under shaking conditions [14]. Therefore, adding an optimum concentration of ethanol in the broth medium results in increasing the BC yields [13, 14, 22]. The addition of ethanol (1.4%, V/V) to the HS medium increased the BC synthesis by about four times that without ethanol [13]. The addition of 1% (V/V) ethanol to the basal medium increased BC production by *G. hansenii* strain from 1.7 to 2.5 g/L reported by Park et al. [23]. The addition of ethanol at a concentration of (1 % v) to a broth culture containing *Komagataeibacter hansenii* cells lead to 80% increase in BC yield [14].

Gamma radiation can be used for enhancing microbial productivity through mutation effect [24]. *K. hansenii* S cell suspension was subjected to low doses (0.2, 0.4, 0.6 and 1 kGy) of gamma radiation to investigate the enhancing effect of these doses on BC production; obtained results revealed that irradiated cells with 0.4 kGy of gamma radiation increased the BC synthesis by about 23% than non-irradiated cells. Mutagenesis has been successfully applied to improve BC production. Mutated *K. xylinus* NCIM 2526 by UV mutagenesis resulted in 30% increase in BC yield [25].

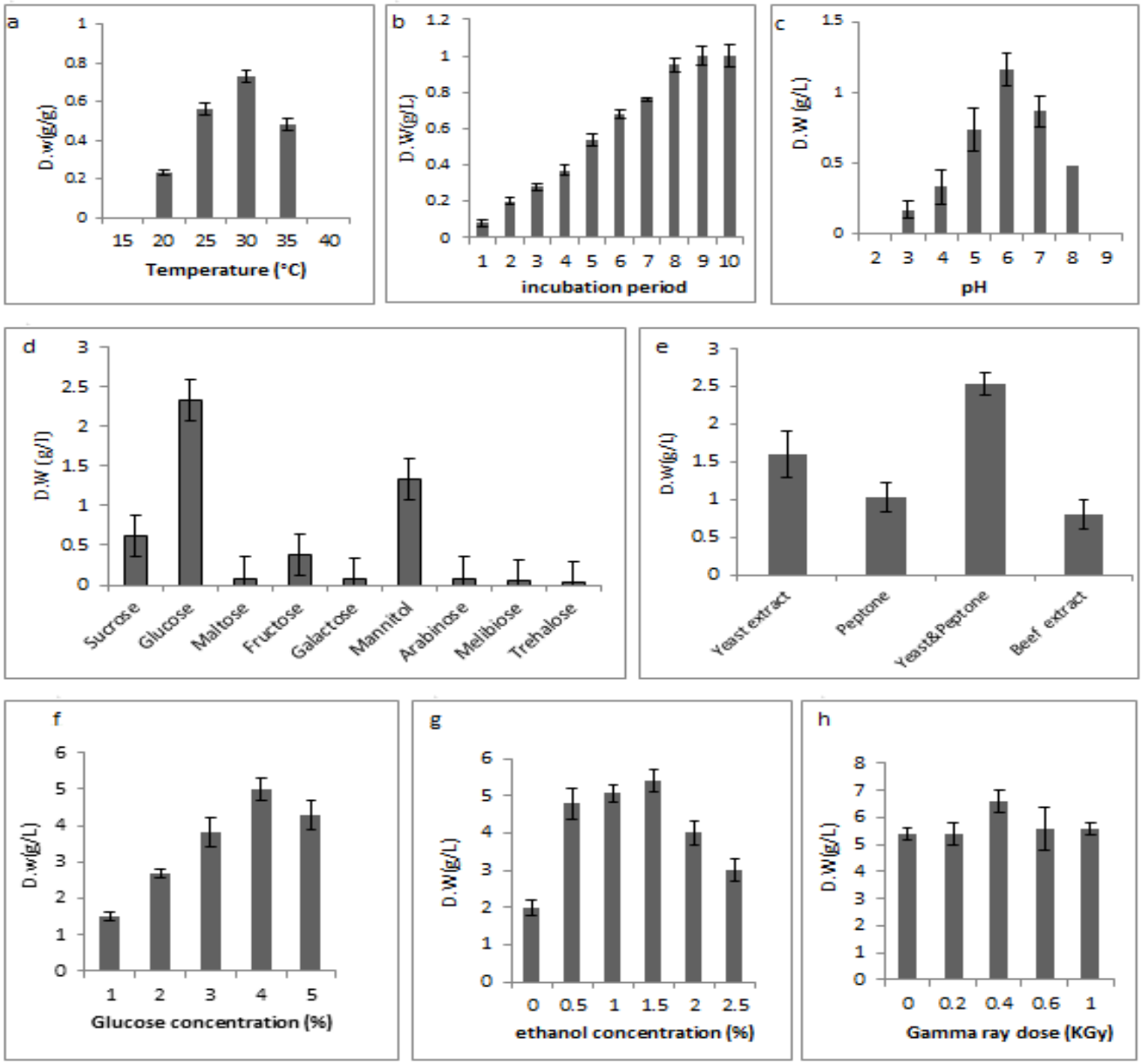

Fig. (2): Effect of different factors (temperature (a), incubation period (b), pH (c), different carbon source (d), different nitrogen source (e), glucose concentration (f), ethanol concentration (g) and different doses of gamma ray (h) on BC production

3.3. Characterization of BC

SEM

SEM images display important visual information on the morphology of the BC (Fig. 3). SEM micrographs clearly display the three-dimensional porous network structure of BC microfibers, especially at high magnification (25000 X).

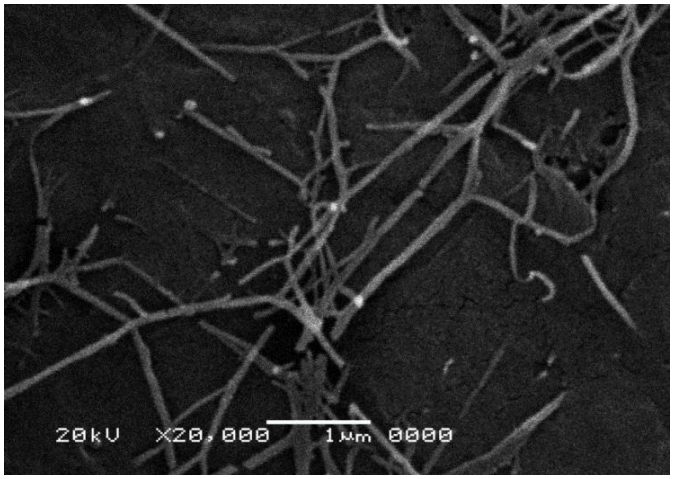

Fig. (3): SEM of BC produced by *Komagataeibacter hansenii* S

FTIR

The FTIR result of BC is shown in Fig. (4). Absorption peaks appeared at the 3359 and 1106 suggesting the presence of a large number of hydroxyl groups (–OH) [5, 26]. Peaks at 2900, 1441 and 667 cm^{-1}, demonstrated the presence of -CH_2- and CH [12, 26]. The band at 1649 cm^{-1} can be assigned to C=O [26]. The band at 1025cm^{-1} is coming from the linear C-O-C [1].

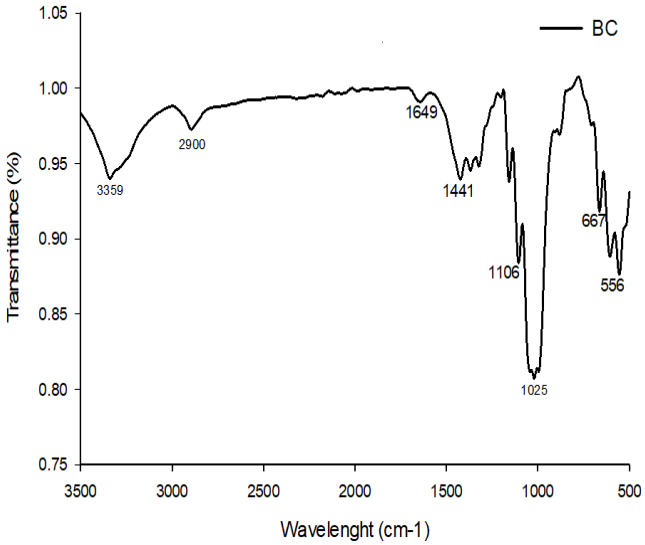

Fig. (4): FTIR pattern of purified BC

XRD

The XRD pattern (Fig. 5) patterns provide information about the crystalline structure of BC, three diffraction peaks appeared at about 14.66°, 16.56°, 22.8° , those peaks were assigned to (110), (110), and (200) crystal planes of cellulose [5]. The apparent crystal size (nm) of BC was calculated according to the Scherrer equation, the peak that was used for calculating the crystalline size of BC is 22.8°. The average size of the BC membrane is determined to be 5.4 nm [27].

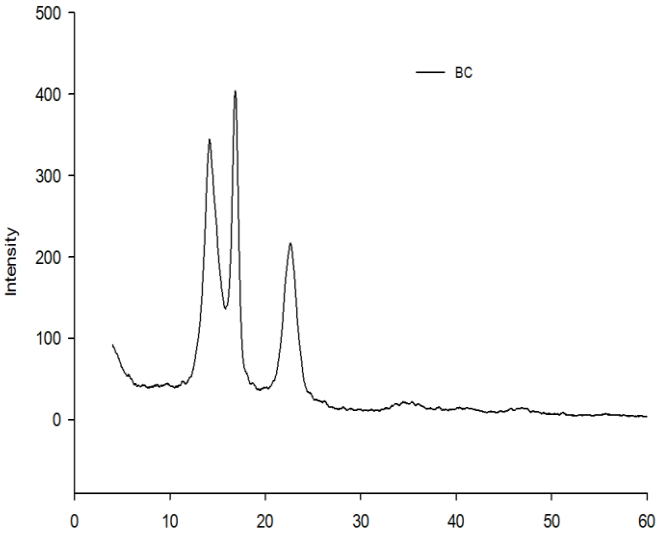

Fig. (5): XRD for purified BC

TGA and DTG

TGA is an important test to know the degradation temperature of a substance. TG and DTG curves of BC show thermal stability. The weight loss under 100°C was possibly due to the loss of water and evaporation of the free moisture content [22]. The bound water in the BC also evaporated but at higher temperatures [28]. A major loss in the weight of BC occurs in the temperature range 280°C to 370°C. The derivative thermogravimetric (DTG) curve (Fig. 6) obtained by thermal degradation of BC pellicle showed the maximum weight loss (maximum rate of transformation) at 320°C indicating a high thermal stability. In another study, the quick drop of a BC sample weight that leads to its decomposition begins at about 300°C, while the maximum rate of weight loss occurred at 350-370°C [29]. Based on TG and DTG results, BC is a suitable material for the construction of fuel cell development because it has a degradation temperature of more than 150°C (the temperature for hot pressing membrane) [30].

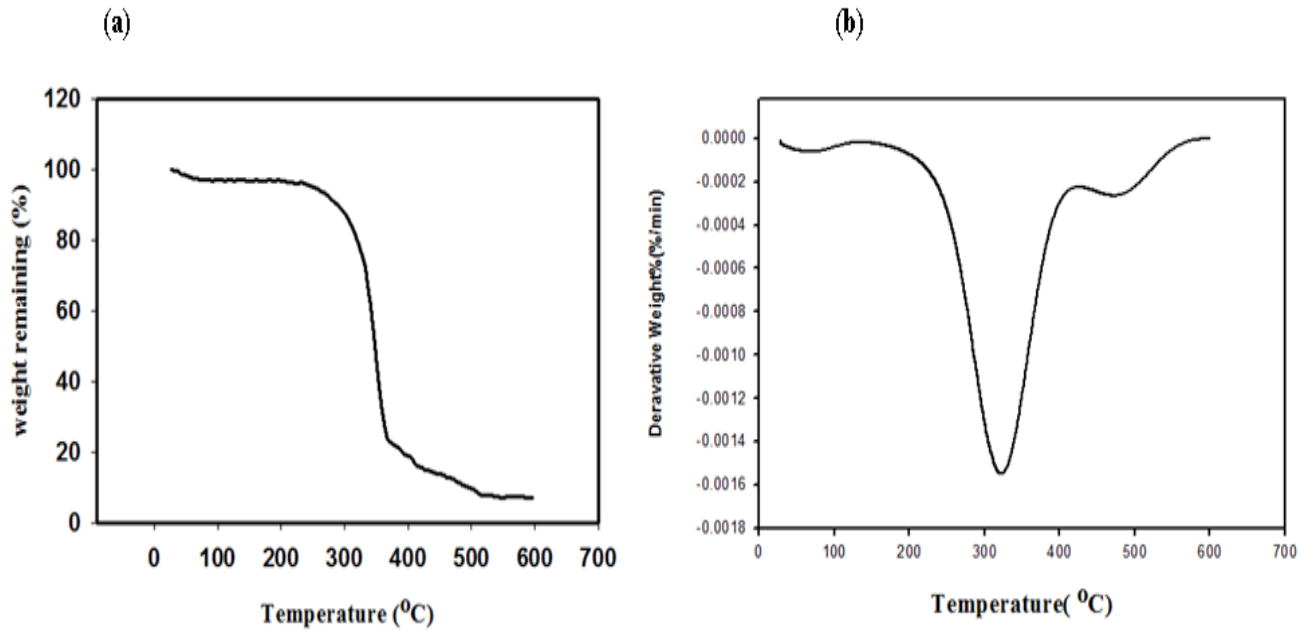

Fig. (6): TG (a) and DTG (b) for BC

DSC

Figure (7) shows an image of thermal transformation obtained by the differential a colorimetry method – DSC. Heat flow curves of cellulose membranes showed an endothermic peak at 155°C for BC produced by *K. hansenii* S, which appeared to be the crystalline melting temperature of the cellulose polymer. According to the literature, at a temperature of 120 – 155°C, there is known to be an occurrence of transformation related to the melting of the crystalline phase of cellulose [31].

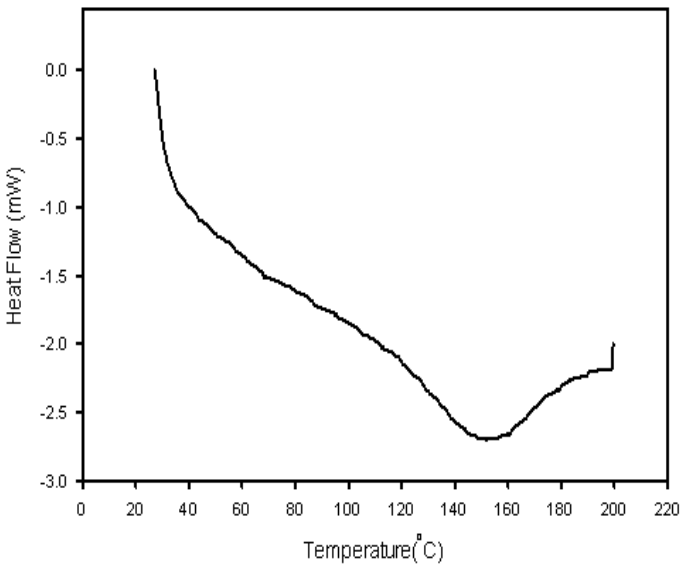

Fig. (7): DSC for synthesized BC

Mechanical properties

Table (2) shows that BC produced by *K. hansenii S in* the present study had good mechanical properties where the values of maximum tensile strength, and max elongation at break were 9.83 MPa, 7.3% and 7.92%, respectively. Our results for mechanical properties of BC were in accordance with many investigators [32, 33, 34].

Table (2): Mechanical properties of purified BC

Mechanical properties	*K. hansenii S*
Max tensile strength (MPa)	9.83
Toughness	17.2
Elongation at max (%)	7.13
Elongation at break (%)	7.92

3.4. Antibacterial activity of amoxicillin/ Flucloxacillin impregnated BC nanofibers

The antibacterial activity of Amoxicillin / Flucloxacillin impregnated BC nanofibers against both gram-positive (*Staphylococcus aureus* ATCC 6538) and Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Klebsiella pneumonia* ATCC 27736 and *Aeromonas hydrophila* ATCC 7965) were studied. It was observed that amoxicillin impregnated BC nanofibers have high antibacterial potency against all tested strains, while BC does not possess any

inherent antimicrobial activity against tested strains (Table 3). The antibacterial activity of BC can be easily achieved by adding antibacterial agents such as antibiotics or metal nanoparticles. The most used antibiotics are amoxicillin, amikacin, ceftriaxone ciprofloxacin, tetracycline hydrochloride (TCH), and vancomycin [35, 36, 37, 38]. There are many advantages of incorporating amoxicillin into BC nanofibers, the first one is that BC has no antibacterial activity to prevent wound infection; incorporating amoxicillin into BC nanofibers provides bioactivity to BC for wound healing, wound dressing, and tissue engineering scaffold. The second one is that amoxicillin is easy to degrade, so it is important to limit the release of amoxicillin to prevent its degradation and loss of its antibacterial potency. BC bilayer film had the ability to retain and slowly release amoxicillin, the three-dimensional network structure of BC nanofiber is the basis for amoxicillin to release continuously. So that BC impregnated amoxicillin could prevent the easy degradation of amoxicillin and represent a good scaffold for amoxicillin [37, 39].

Table (3): Inhibition zone diameter (mm) of amoxicillin/ Flucloxacillin impregnated BC nanofibers and BC

Strains	amoxicillin/ Flucloxacillin impregnated BC	BC
Escherichia coli (ATCC 8739)	25	-
Klebsiella pneumonia (ATCC 27736)	15	-
Staphylococcus aureus (ATCC 6538)	30	-
Aeromonas hydrophila (ATCC,7965)	35	-

CONCLUSION

In conclusion, the results obtained showed that the optimum factors for BC production were achieved at a temperature of 30 C, incubation time of 10 days, pH of 6.0, glucose concentration of 4%, ethanol concentration 1.5%, and gamma-ray dose of 0.4 KGy, where the amount of BC produced is (5.4±0.6 g/l, dry weight). Amoxicillin/Flucloxacillin-impregnated BC nanofibers exhibited a high antibacterial activity against both Gram-positive and Gram-negative bacteria. The physical, chemical, and mechanical properties of produced BC by *Komagataeibacter hansenii* S indicate its availability to be applied in food and medical applications.

Author contributions: All authors contributed equally to this manuscript.

Declarations

Conflict of interest: The authors declare that there are no conflicts of interest.

Ethical approval: This article does not contain any studies with human participants or animals.

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