

ARTICLE / INVESTIGACIÓN

Description and determination of the nanocellulose components produced from acetic acid bacteria

Entissar Faroun Ahmed^{1*}, Wala'a Shawkat Ali¹, Nadhem Hasan Heider²

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¹Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.²Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq.

Corresponding author: entissar.ahmed1102@gmail.com

Abstract: Some microorganisms can produce nanocellulose, which is known as bacterial nanocellulose (BNC); the most active bacterial producer is acetic acid bacteria (AAB), which is a gram-negative, motile and obligate aerobic belongs to the family Acetobacteraceae. Bacterial nanocellulose has excellent attention in medical (surgical domain), industrial and pharmaceutical fields because of its flexible properties, characteristics and advantages. So, in this study, the AAB (5AC) isolate was isolated from apple vinegar. The production of BNC was performed by using a natural medium called palm dates liquid medium, the produced bacterial Cellulose was purified by using the sodium hydroxide method; it was observed that the wet weight of the BNC was about (43.11gm), and its dry weight was about (2.2gm); also the bacterial nanocellulose was characterized by various techniques for detect the morphology of the surface area of it, these techniques are: FESEM technique apparatus which used to clarify the surface morphology of nanostructured bacterial thin films, the results of FESEM presented the presence of different nano-scale diameters of nanofibers. Also, Energy Dispersive X-ray Spectroscopy analysis (EDX) was performed. The results showed that the AAB bacterial cellulose membrane composed of the elements: C, O, H, N and Na (40.71%), (13.98%), (38.6%), (5.41%) and (1.3%) respectively, the FTIR analysis showed that the bacterial nanocellulose fiber functional groups and chemical bonds were observed at the region with wavelength (800-1700) cm⁻¹. All these techniques provide an idea about surface morphology and the composition of BNC membrane, giving it many properties that consider BNC a safe bioproduct in many industrial fields.

Key words: Purification, characterization, nanocellulose, acetic acid bacteria.

Introduction

The progress of nanotechnology in the last twenty years has focused much attention on the electrospinning process; this technique is used to construct nano-polymer important in the bio-medical sector because of its low cost, flexibility and simplicity¹. Cellulose (C₆H₁₀O₅)_n consists of a D-glucose unit at one end and a C₄-OH group at the non-reducing end, while the C₁-OH is the terminating group². Bacterial Cellulose (BC) is an extracellular polysaccharide created by specific bacterial genera of acetic acid bacteria such as *Acetobacter*, *Agrobacterium*, *Gluconacetobacter*, and other bacteria like *Rhizobium*, *Achromobacter*, *Alcaligenes* and *Aerobacter*; it could be characterized as an alternative to plants Cellulose for some fields of applications such as in the medical area, food and many industries³. The (AAB) acetic acid bacteria belong to the family Acetobacteraceae, the (5- 6.5) is the optimum pH for the growth of AAB⁴. Vinegar is the product of acetic acid bacteria metabolism. It is an aqueous solution of acetic acid and other components, and it is consumed as a food preservative and seasoning. This product is the outcome of (2) stages of fermentation. Anaerobic fermentation is the first step, the alcoholic fermentation of sugars into ethanol caused by yeasts, and the second step is aerobic fermentation, the oxidation of ethanol into acetic acid caused by acetic acid bacteria (AAB)⁵. In organic vinegar that does not use preservative agents after opening the bottle, this could encourage the growth of cellulose-producing aerobic bacteria that are not removed entire-

ly in the filtration procedure, which causes the creation of a pellicles layer on the surface medium of the product, which occurs even in conventional vinegar that contains preservative agents if the acetic acid bacteria are not removed ideally in the filtration method before bottling⁶. Also, according to previous research, dates syrup (Alzahdy syrup) was used for BNC production from *A. xylinum* and showed high quantity of produced cellulose⁷. Presently, much research actions and attention are concentrated on the isolation and production of nano-cellulose fibers; the interest in nano-cellulose comes from the fact that the nano-cellulose collects the physical and chemical properties of Cellulose the ability to be chemically modified by a broad range of reactions and its hydrophilicity, with the features, of nanomaterial.

Its high specific surface area and aspect ratio, also the microbial Cellulose has an excellent tensile strength in compared with plant cellulose⁸. The cellulose nanocrystals are prepared by the acid hydrolysis of BC, characterized as: nontoxic, environment-friendly, degradable, edible and biocompatible⁹. The (BNC) exhibits an overall amphiphilic configuration due to the high density of surface hydroxyl groups. In contrast, the hydrophobic interactions are initiated by the crystalline structure and extensive hydrogen bonds in polymer chains; for this reason, their amphiphilic properties could be applied to stabilize surfactant-free Pickering emulsions¹⁰. The Cellulose's poor solubility and low functionality hamper its application in various fields, such as in medical

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and pharmaceutical areas like wound healing and surgical methods; the inter-molecular and intra-molecular hydrogen bonds render the cellulose fibers insoluble in most solvents, different chemical modifications such as cross-linking, or graft-copolymerization, reactions were lately applied to prepare functionalized cellulose¹¹. The produced bacterial nano-cellulose positive outcome of the nano-cellulose membrane or pellicles could be possibly observed as the formation of a gel-like mat that floats on the medium surface; this (BNC) produced by the bacterial isolate could be purified by (0.1N) of NaOH¹². The structure of BNC, such as the determination of width, length, cross-linking, and chemical compositions of the Cellulose, could be characterized by using some methods like scanning electron microscopy (SEM), Energy Dispersive X-ray Spectroscopy (EDS) and Fourier transform infrared spectrophotometer (FTIR). Also, other essential parameters characterize the (BNC) such as the degree of polymerization (D, P) and crystallinity, which is measured by the XRD techniques¹³. In the present study, BNC, produced from acetic acid bacteria (AAB) isolated from vinegar, was purified and characterized.

Materials and methods

Bacterial isolates

The acetic acid bacteria (AAB) were isolated from apple vinegar and date palm vinegar using three culture media: GYC broth medium¹⁴, GY broth medium, and GYC agar medium¹⁵. A total of 25 samples from different types of vinegar were collected under aseptic conditions from the local market in Baghdad city. About 5 isolates were characterized as AAB from the total samples according to procedures of (14,16), and the identification of bacterial isolates was confirmed by the Real-Time Polymerase chain reaction (PCR) amplification technique¹⁷, the bacterial isolate (5AC) was selected as BNC producer after screening.

Production medium

Palm dates liquid medium (pH= 6) was used as production medium, and it was prepared by mixing 15gm of palm dates (kind: Osta-Omran) waste with 100 ml of DW; this palm dates waste was blended by blender. After that, sifted in a sieve and then 1gm of glucose was added to each screwed cup of the medium¹⁸.

Production of BNC from AAB:

A total of one liter of the production medium (palm dates liquid medium), was inoculated with overnight activated bacterial isolate (5AC) culture broth (the inoculum size 2%) and incubated at (30°C) for (8-10 days), after this period, the BNC was performed in the medium, the (BNC) wet weight and dry weight then were determined.

Purification of (BNC) produced by Acetic acid bacteria (5AC):

The layers of (BNC) that formed, at the air-liquid interface in the production medium: (palm dates liquid medium) were taken and washed with water and soaked in (0.1M) NaOH at 80°C for (30 to 45 min) to remove the bacterial cells that probably attached to the BNC membrane. Then, the membranes were washed with distilled water several times to confirm the whole removal of the alkali; the membranes were left at neutral pH. The purified Cellulose was dried

at room temperature until reaching a constant mass^{3,19}.

Characterization of bacterial nanocellulose BNC:

After the purification and drying BNC membrane that formed by AAB (5AC), isolate, this dried membrane was characterized by:

Field Emission Scanning Electron Microscope (FESEM)

A piece of BNC layer was placed on a glass slide and allowed to dry, and then, a thin layer of gold was coated in the sample of BNC layer^{20,21}. The surface morphology and composition of thin films of this test were carried out in Alkhora company SEM lab/Baghdad using FEI (Inspect™ F50) Field Emission Scanning Electron Microscope (FESEM) which was operated at a vacuum system; the operated accelerating voltage was kept in the range 200V to 30 kV.

Energy Dispersive X-ray Spectroscopy Scanning Electron Microscope (EDS-SEM or EDX-SEM)

To determine the elements composition of the sample, a piece of purified BNC layer and a small amount of manufactured cellulose suspension (as a standard) were placed on glass slide samples and left to dry, then analyzed by EDS-SEM device²¹.

Fourier Transform Infrared Spectrophotometer (FTIR)

The characterization of functional groups of the BNC and the powder of Cellulose were investigated by FTIR analysis instrument (FTIR- 8400/Shimadzu-Japan), and the spectra were scanned in the range of (400–4000) cm⁻¹. The samples were prepared by uniformly dispersing the dried BNC and the standard cellulose powder in a matrix of dry KBr in a mortar and grinding to mix well, then compressed to form an almost compact disc in a specific device under vacuum. KBr was used as a standard to analyze the samples²¹.

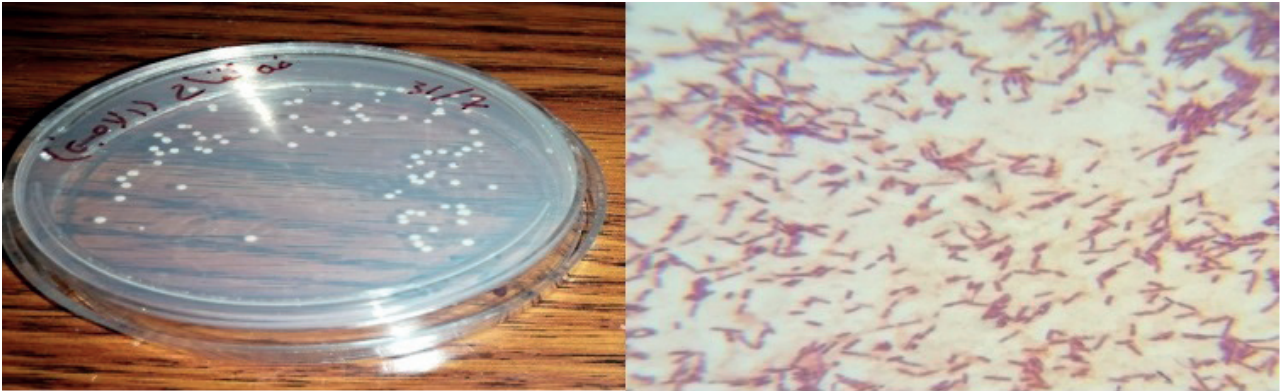
Results and discussion

Identification of Bacterial Isolates

Twenty-five samples were collected from different vinegar types: (apple and palm date vinegar), five isolates were characterized as acetic acid bacteria (AAB) from the total samples. The bacterial colony was aerobic, round, smooth, raised with white or off-white, and semi-translucent opacity. Microscopic examination of the isolates: displayed that they were all: Gram-negative, non-spore-forming and rod, (Figure 1). Results of biochemical tests showed that all isolates are negative for oxidase and positive for catalase, motile, and produce Cellulose. The results of the Real-Time PCR amplification technique confirmed the identification of these five isolates as acetic acid bacteria (AAB). The acetic acid bacteria (AAB) are Gram-negative, rod-shaped and can occur as a single cell, in pairs or chains; they are motile due to the presence of flagella which can be either polar or peritrichous; they do not form endospores; they have an obligate aerobic metabolism, that the oxygen as terminal electron an acceptor^{23,24}.

Production of BNC from AAB

After incubation of the AAB (5AC), a bacterial isolate



-A-

-B-

Figure 1. A- Acetic acid bacterial cells under the microscope (100X), B- Colonies of acetic acid bacteria (AAB) on GY agar medium.

that can produce bacterial nanocellulose (BNC) for (8-10) days in palm date liquid medium, the BNC was observed as a white pellicle with a diameter of approximately 48 mm covering the surface of the liquid medium (Figure 2), it was found that the (1Liter) of medium gave about (55.41gm) wet weight of BNC. *Komagataeibacter xylinus* (formerly *Acetobacter xylinum*) is the most generally used species of bacteria for the production of bacterial Cellulose (BC) since it produces quite large amounts of this bacterial cellulose from a wide range of carbon and nitrogen sources in broth culture media²⁵. For the production of BC, carbon sources commonly used are agro-industrial wastes, for example, rotten fruit like pineapple peel juice, and sugar as a medium used to yield a quantity of bacterial cellulose²⁶. The yield of bacterial cellulose synthesis is up to (40) %, in relation to the starting carbon source in the large scale production of BC⁸. The researchers found that the production of the bacterial nano-cellulose (BNC), representing, the assimilation, of sugars within the bacterial cells and the creation of β -1,4, glucan chains to lastly construct, the micro-structure of Cellulose, actually the BNC generated on the wastes primarily presented analogous morphological features to the control BNC mainly after washing¹². Bacterial Cellulose could be industrially, produced and broadly, used in various, areas.. Doninii *et al.* (2010)²⁷ value that the, fer-

mentation production of the bacterial Cellulose could achieve, a similar production, efficacy with the growth, of plant cellulose, when the yield of bacterial Cellulose reaches up to (15) g/L in,500 hr²⁸.

Purification of BNC produced by (5AC) Acetic acid bacteria

After the production of bacterial nanocellulose in (1L) of natural medium (palm date liquid medium), the BNC layers were harvested and purified by sodium hydroxide (0.1M NaOH). The wet weight of the BNC was measured after the purification and it was found about (43.11gm), while the dry weight of it was about (2.2gm) (Figure 3).

The BNC isolation and purification are relatively simple and do not need wide chemical or any other types of treatments, in contrast with wood cellulose and plant cellulose²⁹. The BNC membrane was separated and purified from the treatment medium with distilled water. (DW) and weak alkaline treatment of (0.5)M sodium hydroxide, where it was possible, to dry these membranes and characterize them by various techniques to investigate the membrane composition, crystallinity, and its fibrils morphology,, the results obtained from purification were acceptable, decreasing, the water band, of the biomaterial, increasing the crystallinity and also eliminating impurities, in the membrane such as: a

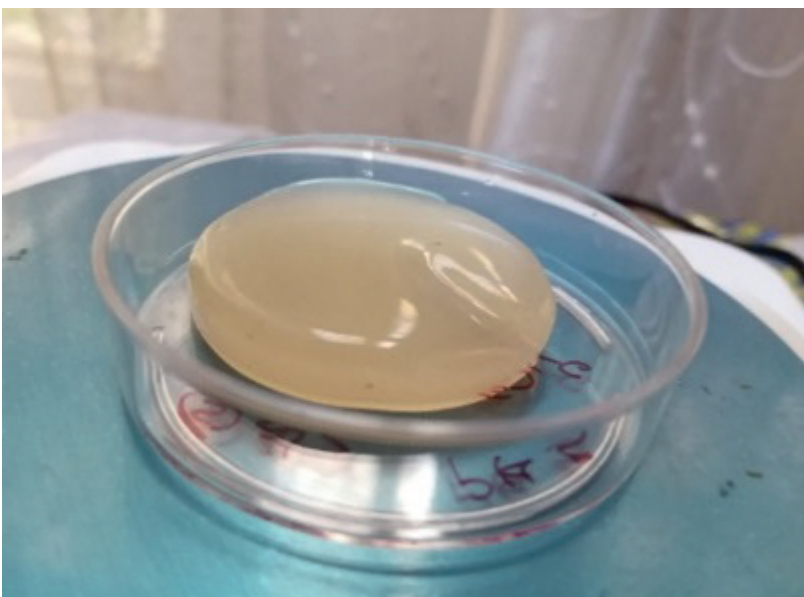


Figure 2. Bacterial nano-cellulose membrane: (wet membrane).



Figure 3. Bacterial nanocellulose membrane (dry membrane) of (5AC) Acetic acid bacteria.

bacteria and other, stuck materials and also with, the purification that performed, it will be possible to more additional study of the material for use as a product such as in: biomedical field and tissue engineering as it used as a biomaterial reinforcement, a (30). The BNC purification using (1M) of sodium hydroxide ((.NaOH) solution is effective. And considered as eco-friendly with no signs of recalcitrant development as commonly found in plant cellulose purification steps³¹.

Characterization of BNC, by Field, Emission Scanning Electron, Microscope (FESEM),

(FESEM) was used to clarify the surface morphology of nanostructured thin films. A cross-sectional view of the films could be taken, with the help of FESEM to get information about the thickness of the experimented film and also characterize the particle diameter in addition to the morphology of nanoparticles²². The morphology of the BNC membrane, a was analyzed by the FESEM system. This technique of scanning electron microscope showed that the BNC consists from net of very fine fibers which made together the whole mat of bacterial Cellulose, the average diameter of the bacterial cellulose fibril was approximately (38.56 nm) (Figures 4 and 5).

The bacterial Cellulose structurally appears in SEM images as ribbon-like cellulose nanofibers that further in-

terweave into 3D, reticulated net, so that it was characterized with high purity, high crystallinity and also high degree of polymerization²⁸. The structure of Cellulose produced from bacteria (BC.) is the form of fiber with nano-sized width, analogous to silk, natural fiber formed from the silkworm; this bacterial Cellulose tends to be produced as nano-sized particles with high crystallinity during enzymatic or acidic methods, and these procedures are involved into the "gamification" of the bacterial Cellulose³². The SEM technique was used to study the surface morphology of the bacterial Cellulose, the BC dried samples were sputter, covered with a thin layer of evaporated gold, and a typical mesh of bacterial cellulose fibrils net was detected in the SEM images showed that the average fibril, diameter, of the bacterial Cellulose is 71 nm, which is a nanomaterial³³. The bacterial cellulose membrane structure produced by *K. maltaceti* was examined by SEM analysis, the results showing that the bacterial cellulose membrane synthesized by *K. maltaceti*, after the cultivation, in HS medium at (30) °C, for about (21) days, this BC membrane consist of fibrils were condensed together into a reticular, or net structure with, porous size approximately (50–250), nm²⁴.

Characterization of BNC by EDS

For detection and determination the elements compo-

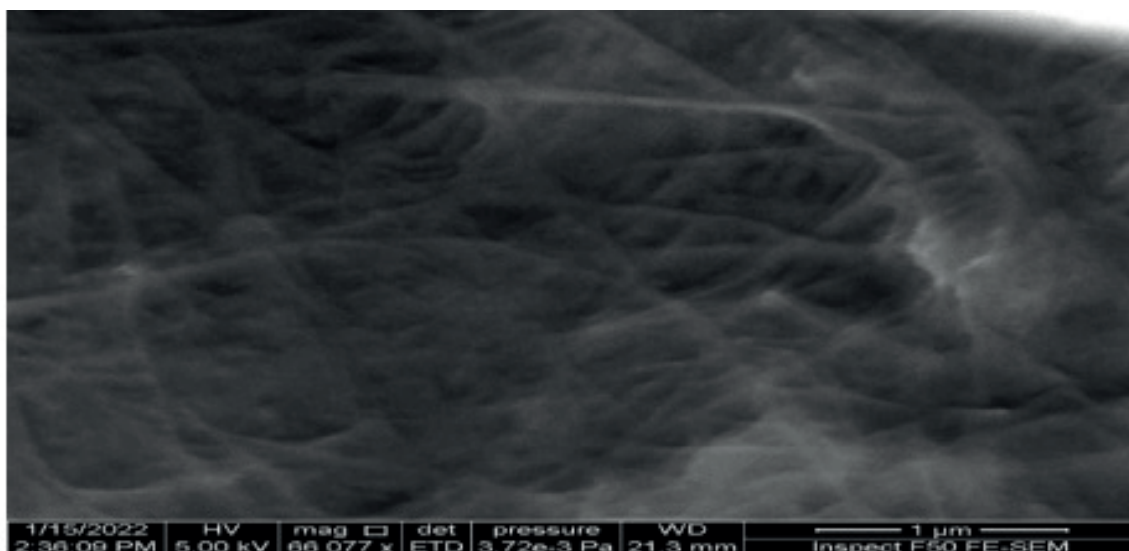


Figure 4. Bacterial nanocellulose (BNC) under Field Emission Scanning Electron Microscope (Top view).

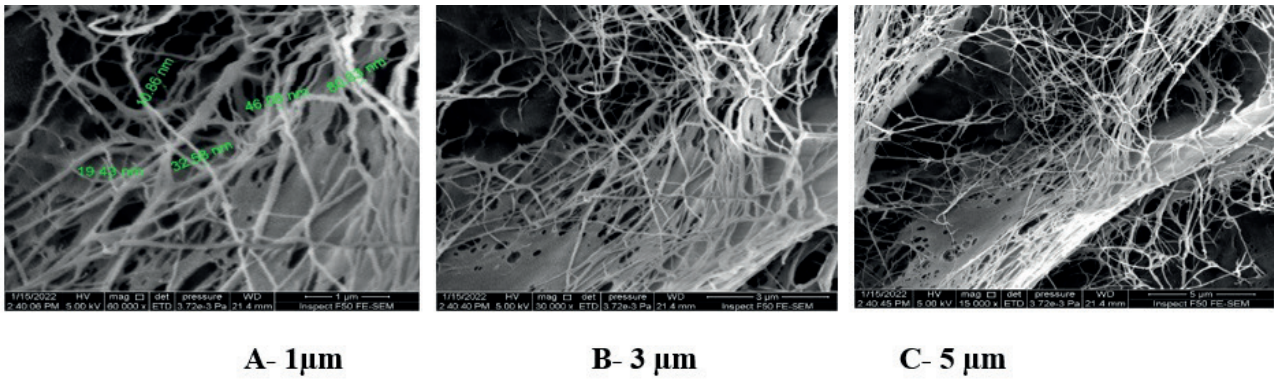


Figure 5. BNC under, Field Emission Scanning Electron Microscope (FESEM) (Cross section of BNC).

sition of the bacterial dried (BNC) products from the acetic acid bacteria isolate (5AC), the Energy Dispersive X-ray Spectroscopy (EDS-SEM) or EDX device was used, the results showed that AAB bacterial cellulose membrane composed of the elements: C (40.71%), O (13.98%), H (38.6%), N (5.41%) and Na (1.3%) as observed in (Figure 6) and as the results showed for the percentages of elements components of bacterial Cellulose which is could be attributed to the components of the medium that the bacterial isolate grown in it and the purification procedure for the bacterial cellulose membrane by (NaOH).

EDX spectroscopy was carried out to confirm the presence of the elements belonging to the functional groups for the Cellulose produced by *Gluconacetobacter xylinus*; the material was described using solid-state techniques be-

cause of the insolubility of Cellulose in common solvents as carbon and oxygen were already present in the structure of Cellulose, *G. xylinus* BC pellicles identified that the carbon content approximately (34.2%), oxygen content is about (38.35%) and the nitrogen content about (0.29%)³⁴.

The BNC produced by *Gluconacetobacter xylinus* was analyzed by scanning electron microscopy (SEM) and for detection of the element's content using energy dispersive spectroscopy (EDX analysis), the elemental results disclose the basic component of BNC with their particular mass and atomic distribution within the BNC sample, the results are shown that the carbon content about (72.124%) and the oxygen content approximately (18.202%)³¹.

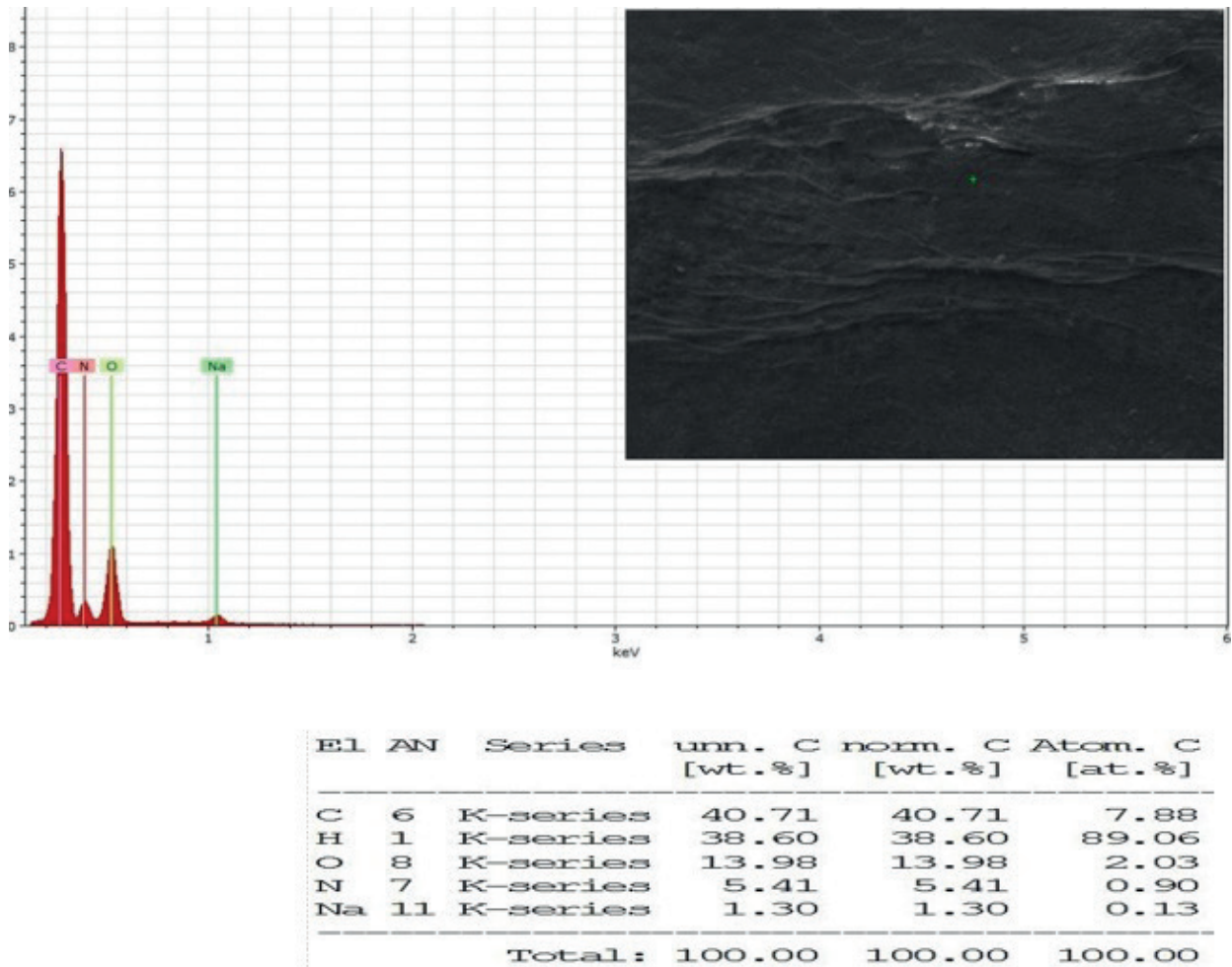


Figure 6. Energy dispersive X-ray spectroscopy (EDX-SEM) elemental mapping image of BNC from AAB (5AC) isolate.

Characterization of BNC by Fourier transform infrared spectrophotometer (FTIR)

The FT-IR analysis was performed in order to determine the functional groups of bacterial nanocellulose produced from acetic acid bacteria (5AC) isolate, the FTIR recorded for dried cellulose membrane sample at a wavelength range of 4000–400 cm^{-1} , the bacterial nanocellulose fiber functional groups and chemical bonds was observed at the region with wavelength (800–1700) cm^{-1} very close peaks values with cellulose peaks values as showed in (Figure 7).

FTIR measurements were used in order to detect the presence of various functional groups in nanoparticle materials by using FTIR, spectrometer, FTIR spectra recorded in the solid case using the (KBr) pellets mechanism in the range of (4000–400) cm^{-1} ^{35,36}. These peaks (3431.13 and 3434.98) cm^{-1} may be expressed to the O-H stretching vibration mode³⁷. The region with wavenumbers 800 and 1200 cm^{-1} supposed to be the fingerprint region for the carbohydrates also the wide vibration band at wavenumbers (3200–3400) cm^{-1} region could be allocated to the (OH) stretching vibration of the hydroxyl groups existing in the bacterial cellulose net fiber, the two comparatively smaller bands at (2800–2900) cm^{-1} may be considered to the stretching vibrations of CH, the peak at 1158 cm^{-1} was resultant from the (C–O–C) stretching vibration of the bacterial cellulose matrix³⁸. The wavenumber of the peaks at the area of 1200–900 cm^{-1} ascended from the C–O and C–C stretching vibrations of the cellulose net fibers; the difference in the strengths of various bands could be due to the diverse thickness of the cellulose samples that used in the measurements of Fourier transform infrared spectrophotometer (FTIR)^{38,39}. Due to the importance of the bacterial isolates, it was confirmed by (RT-PCR) amplification technique.(17) the molecular technique applied in different areas related to biology^{40–62}.

Conclusions

The bacterial nanocellulose (BNC) synthesized by ace-

tic acid bacteria (AAB) was efficaciously performed under cultural conditions using a natural medium, which is palm date liquid medium (pH 6) that was used as a production medium that incubated conditions at 30°C for (8–10) days. The BNC was purified by sodium hydroxide method, the BNC was described and different techniques determined its components, these techniques are: FESEM which used to clarify the surface morphology of nanostructured bacterial thin films, Energy Dispersive X-ray Spectroscopy (EDS) for detection and determination the elements composition of the bacterial dried (BNC) products and Fourier transform infrared spectrophotometer (FTIR) was performed in order to determine the functional groups of bacterial nanocellulose produced from acetic acid bacteria. The results from all these techniques and analyses gave the BNC more attention in many fields: medical, industrial and electrical applications, because of its valuable properties such as its nanofibers having a high ability to absorb liquids and also its high tensile strength and many other characteristics that make it essential in many applications.

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Conflicts of Interest

The authors declare no conflict of interest.

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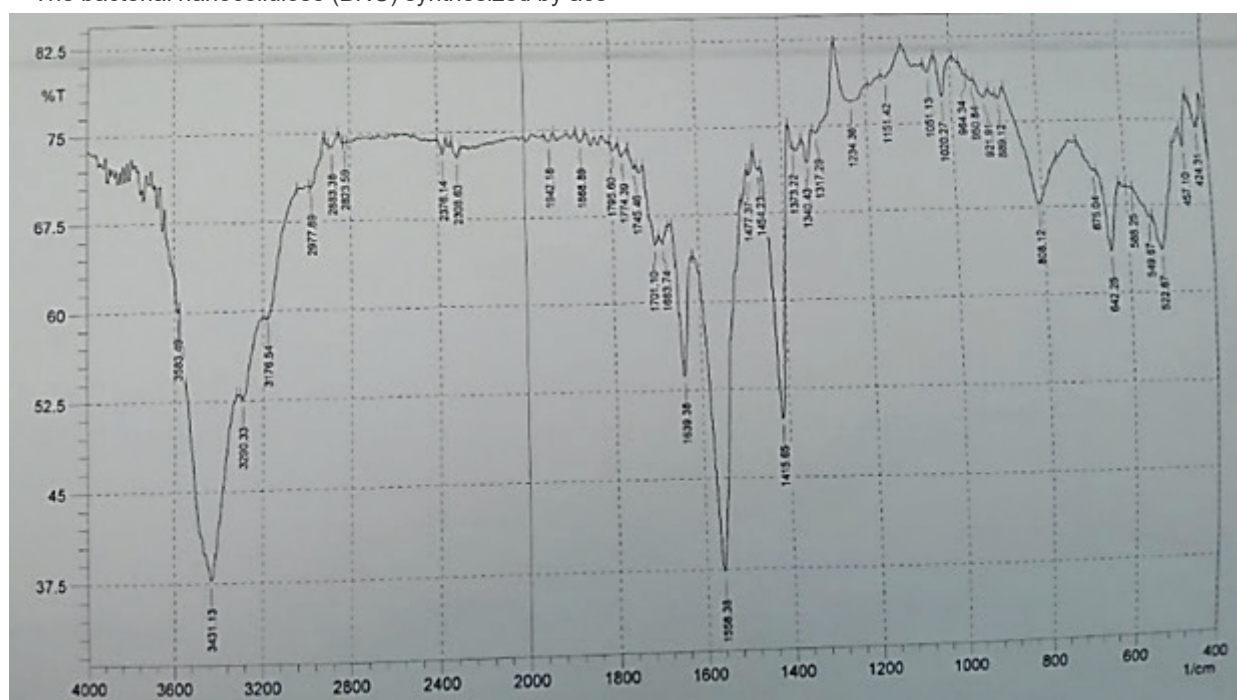


Figure 7. FTIR analysis for BNC produced from acetic acid bacteria (5AC) isolate.

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