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Investigation of Physicochemical Properties of the Bacterial Cellulose Produced by *Gluconacetobacter xylinus* from Date Syrup

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***Abstract*—**Bacterial cellulose, a biopolysaccharide, is produced by the bacterium, *Gluconacetobacter xylinus*. Static batch fermentation for bacterial cellulose production was studied in sucrose and date syrup solutions (Bx. 10%) at 28 °C using *G. xylinus* (PTCC, 1734). Results showed that the maximum yields of bacterial cellulose (BC) were 4.35 and 1.69 g/l00 ml for date syrup and sucrose medium after 336 hours fermentation period, respectively. Comparison of FT-IR spectrum of cellulose with BC indicated appropriate coincidence which proved that the component produced by *G. xylinus* was cellulose.

Determination of the area under X-ray diffractometry patterns demonstrated that the crystallinity amount of cellulose (83.61%) was more than that for the BC (60.73%). The scanning electron microscopy imaging of BC and cellulose were carried out in two magnifications of 1 and 6K. Results showed that the diameter ratio of BC to cellulose was approximately 1/30 which indicated more delicacy of BC fibers relative to cellulose.

Keywords*—Gluconacetobacter xylinus*, Fourier Transform Infrared spectroscopy, Scanning Electron Microscopy, X-ray diffractometry

I. Introduction

CELLULOSE forms the basic structural foundation of the cell wall of eukaryotic plants and algae and is also found as a major constituent of the cell wall of fungi [3]. It is therefore, the most abundant bio-polymer on the earth with 180 billion tons per year produced in nature [8]. A simple straight chain polymer of glucose molecules linked at the β, 1-4 position, and cellulose is the most commonly harvested from trees and cotton but is also derived from flax, jute and hemp. Bacteria also synthesize cellulose, including the genera Agrobacterium, *Rhizobium, Psuedomonas, Sarcina and Acetobacter* [3].

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Acetobacter xylinum (or reclassified *Gluconacetobacter xylinus*), a Gram-negative, rod shaped bacteria and a prodigious producer of cellulose, has been studied extensively. Initially, the extra-cellular cellulose produced by the bacteria was seen as a method for elucidating the biosynthetic pathway of cellulose, but bacterial cellulose has developed into a field of study of its own. As opposed to cotton and paper, where the purification of the cellulose product decreased the chain length, bacterial cellulose does not require remedial processing to remove unwanted polymers and contaminants (e.g. lignin, hemicellulose) and therefore, retains a greater degree of polymerization [15]. This fact gives bacterial cellulose superior unidirectional strength. In a native state, bacterial cellulose also has a greater water holding capacity over a hundred times its own weight in water. These properties, along with the *in situ* ability to mould the cellulose during production, have led to innovative uses for bacterial cellulose. Included among the uses are high quality speaker diaphragms, membranes, a food bulking agent, medicinal bandages and potentially as replacement blood vessels [18]. To make the subject more confusing, it has been established that there are four structurally different types of cellulose, with different properties that have been identified: cellulose I, consists of β, 1-4 glucan chains aligned in parallel, typically found in nature. Cellulose I is the cellulose which is produced in pellicle form by *G. xylinus*. In an undried state, cellulose I may be referred to as "native" cellulose [16]. Cellulose II, consists of anti-parallel β, 1-4 glucan chains, found in shaken culture of *G. xylinus* or after re-crystallization or industrial mercerization of cellulose I [16]. Cellulose III, consists of chemically treated cellulose I [9]. Cellulose IV, found in cell wall of higher plants and can be derived from chemically treating cellulose II [9]. It has been only in the last two decades that key advances have been made into the cellular activators that cause *G. xylinus* to secrete cellulose. Cellulose I (alpha) was produced predominately by bacteria and algae while cellulose I (beta) was derived from plants. However, all native cellulose contains quantities of both allomorphs [21]. This suggested a model orientation of the cellulose chains with I_α containing a triclinic unit cell and I_β containing a monoclinic unit cell. Furthermore, silver labeling proved that all the reducing groups were orientated the same way [11]. Bacterial Cellulose (BC) has gained attention in the research realm for the favorable properties it possesses; such as its remarkable mechanical properties in both dry and wet states, porosity, water absorbency, moldability, biodegrability and excellent

biological affinity [19]. Because of these properties, BC has a wide range of potential applications including use as a separation medium for water treatment [2], [4], a specialty carrier for battery fluids and fuel cells [2], a mixing agent, a viscosity modifier [2], [12], immobilization matrices of proteins or chromatography substances [12], [20], etc. The prevalent application of BC is in the biomedical field, as it is highly useful for wound dressing [10], [5], [14], [22], [6]; artificial skin [12], [7]; dental implants; vascular grafts; catheter covering dressing [22]; and as artificial blood vessels [13], [1], [23]. In this study, bacterial cellulose was produced by *Gluconacetobacter xylinus*. The chemical structure was characterized by Fourier Transform Infrared Spectroscopy (FT-IR). Changes in the physical properties were observed using scanning electronic microscopy and X-ray diffraction.

II. Materials And Methods

A. Preparation of date syrup

The initial extract was prepared by soaking 200 g of stone free, low quality date fruits in 500 ml distilled water then mixed in a blender for 1 min at low speed, and for additional 3 min at a high speed. The homogenized extract was filtered through a double layer of cheese cloth. The residue was then washed with hot water and solution made up to the volume required to the make concentration of 20 %; subsequent dilutions to Brix 10 were made with distilled water. To remove all insoluble solids, the date extract solutions were centrifuged (Sorvall, RC-5; USA) at 10000×g for 30 min at room temperature.

B. Stock Culture

The organism exploited for the production of cellulose in this work was a strain of *Gluconacetobacter xylinus*, which was obtained from the Persian Type Culture Collection (PTCC), strain number 1734. The microorganisms were maintained in the test tubes containing tomato serum medium and were transferred monthly. The typical composition of the stock culture medium was 50 g/l glucose, 5 g/l bactopeptone, 5 g/l yeast extract and 10% by volume tomato juice at pH, 6.8. The stock cultures were stored at 5˚C to slow down the growth and cellulose production.

C. Inoculum Preparation

A culture medium composed of 5% (w/v) glucose, 0.5% bactopeptone, 0.5% yeast extract, 0.27% sodium phosphate monobasic and 0.12% citric acid was used in all cellulose production experiments. About 200 ml of this medium in 500 ml Erlenmeyer flasks was autoclaved at 121˚C for 15 minutes prior to inoculation. After cooling to room temperature, the flasks were inoculated with the stock culture and incubated in a Benmarin shaker (240 rpm) set at 28 ˚C and pH, 6.8. The organisms were collected by centrifugation (2500×g, 20 min) and resuspended in liquid medium and the bacteria were inoculated into two liquid fermentation media in Erlenmeyer flasks containing sucrose or date syrup.

D. Cellulose production

The procedure for production of bacterial cellulose is shown in Figure 1. *G. xylinus* was grown in a generic medium derived by Schramm and Hestrin [17] and its composition is shown in Table I. After preparation of inoculums, organisms were inoculated into two liquid fermentation media in Erlenmeyer flasks containing sucrose or date syrup (Brix 10) as a carbon source. Fermentation occurred in static state set at 28 ˚C and pH, 6.8. Samples were taken from fermentation media after 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 336 and 360 h. For all experiments triplicate flasks were prepared for each treatment. After growth and centrifugation, pellets were removed and analyzed in triplicate for their cellulose content.

E. Yield of cellulose production

The bacterial cellulose produced during the course of the fermentation was measured at the end of each run. The bacterial cellulose was normally washed with water to remove any residual sugar. The film was then boiled in 2% NaOH solution for 1 h to remove cells from the cellulose matrix. The cellulose was washed with deionized water until the remaining base was removed. The dry weight was measured after drying the film for three days at 45˚C. The dried bacterial cellulose samples were weighed, and values were converted to mg/100 ml of the original medium.

F. Statistical data analysis

One-way ANOVA analysis by SPSS 17 software performed for yield data analysis. Factorial test carried out to indicate simultaneous effect of the treatments (date syrup and sucrose media) and production time. Duncan test at level of 0.05 was accomplished for explanation of production time's effect on yield.

G. Pellicle production and purification

Bacterial cellulose was produced by *G. xylinus* in 2000 ml Erlenmeyer flask at 28 ˚C for 30 days. After incubation, the pellicle produced on the surface of the media containing date syrup was separated. For removal of the microbial cells, the pellicles were washed with water, 4 % (w/v) sodium hydroxide solution in boiling bath (30 min), 6 % (v/v) acetic acid, and then water, successively.

H. Fourier Transform Infrared Spectroscopy (FT-IR)

The samples (Pure cellulose and Bacterial cellulose) were well mixed with potassium bromide (KBr) powder and pressed into a small tablet. FT-IR spectra were recorded using a Brucker spectrometer (EQUINOX55, Germany) in the transmittance mode with a resolution of 1 cm^{-1} in the range of 4000-400 cm^{-1}.

I. Scanning electron microscopy (SEM)

Thin layers of the samples were coated with gold using an ion sputter (Fisons Instruments, UK). The coated samples

were viewed and photographed using the Scanning electron microscope (model 5526, Cambridge, UK) at 20 kv.

J. X-Ray analysis

To determine the crystallinity of the two samples, the X-ray diffraction (XRD) patterns of the samples were collected on a Siemens (D5000-Germany) Standard Theta/2Theta diffractometer using a copper x-ray source. Scans were collected at 2 degree per minute from 5–60 degree 2θ. The samples were lyophilized overnight using a lab-scale freeze drier (Armfield model) and pressed into a thin and flat layer (~1.5 mm thickness) for analysis.

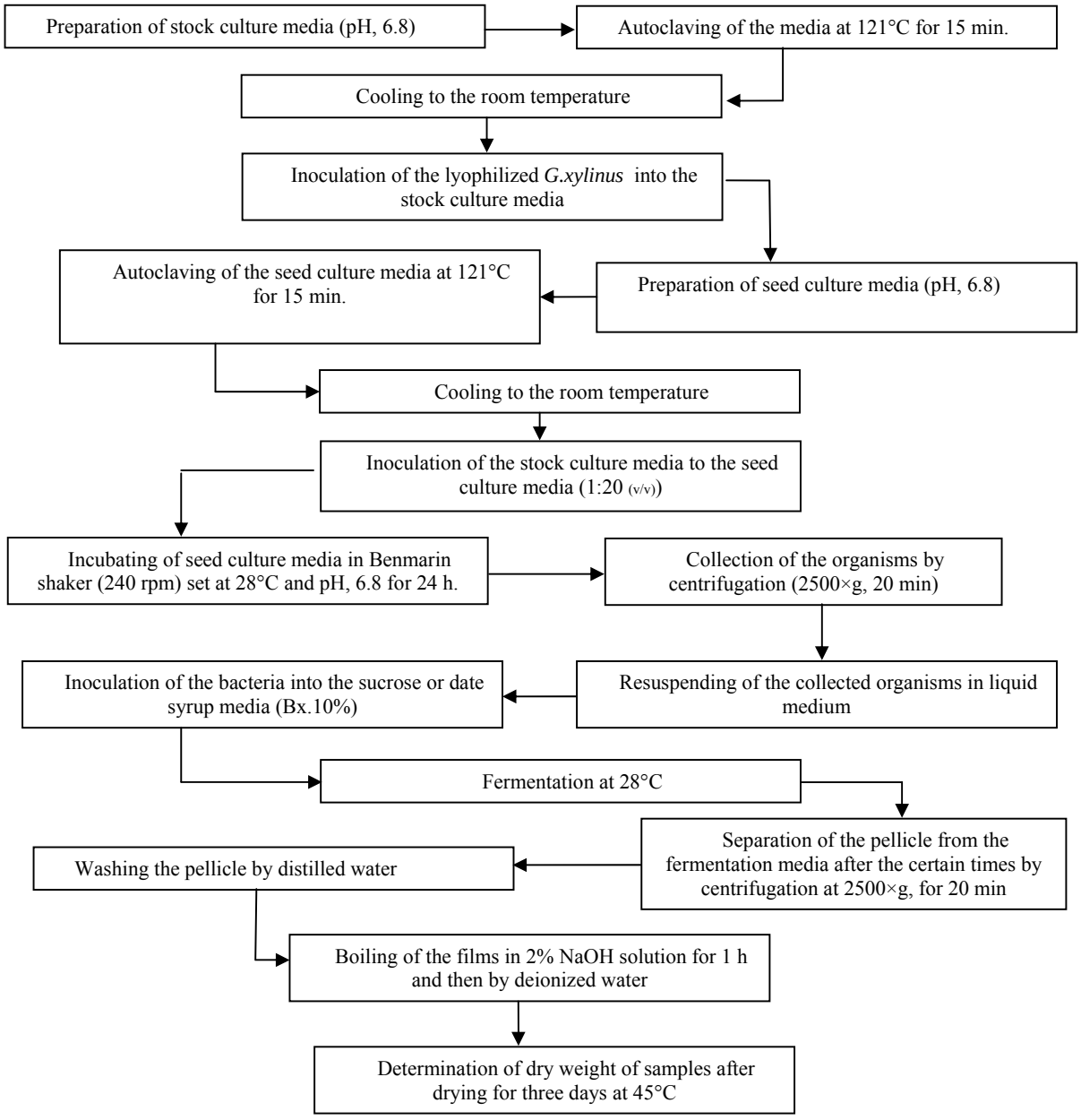

Fig. 1 Schematic diagram of the procedure for bacterial cellulose production.

TABLE I
THE COMPOSITION OF THE GENERIC MEDIUM
USED AS SEED AND FERMENTATION CULTURE MEDIA

Component	Quantity
Carbon source (Brix 10)	1 L
Na_2HPO_4	2.7 g
Bactopeptone	5 g
Yeast extract	5 g
Citric acid	1.5 g

III. RESULTS AND DISCUSSION

A. Yield of bacterial cellulose production

The rate of bacterial cellulose production in fermentation media containing sucrose or date syrup were monitored over a period of 360 h incubation at 28 ˚C in static state and the results are given in Figure 2. The BC production in the fermentation medium containing sucrose relatively remained constant but at the fermentation medium containing date syrup, biogum production continuously increased until the 14th day of fermentation period. *G. xylinus* in fermentation medium containing date syrup gave a significantly ($p<0.05$) higher yield of BC than fermentation medium containing sucrose. BC production using date syrup was 1.5 to 2 times higher than the medium containing sucrose during fermentation period. The highest yield of production obtained after 14 days incubation at 28 ˚C for either date syrup or sucrose fermentation media.

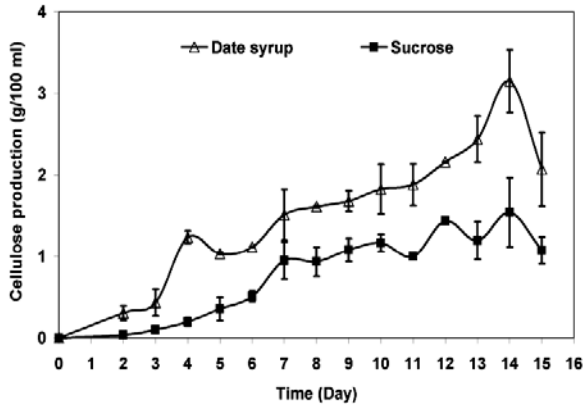

Fig. 2 Yield of bacterial cellulose production in fermentation media containing either date syrup or sucrose during fermentation

B. ANOVA test analysis

The yields from each sampling time were tested by Duncan. The obtained results showed four non-significant groups that are shown in Table II. These results demonstrated that the yield after 336 h fermentation was significantly different relative to all the other yields at the level of 0.05. Factorial test (time × treatments (date syrup and sucrose media) × repetition) results (Table III) represented the significant difference for treatment and sampling times alone at the level of 0.001 while time × treatment interaction did not show any significant difference even at the level of 0.05.

TABLE II
RESULTS OF DUNCAN TEST FOR YIELDS OF PRODUCTION

Time (h)	Weight (g/ml)	Time (h)	Weight (g/ml)
48	0.17075^{a}±0.1638	216	1.38^{bcd} ±0.3634
72	0.267^{a} ±0.2134	240	1.495^{cd} ±0.5289
96	0.717^{ab} ±0.4005	264	1.44^{bcd} ±0.4239
120	0.6945^{ab} ±0.6017	288	1.795^{d} ±0.9477
144	0.812^{abc} ±0.3520	312	1.9425^{d} ±1.9834
168	1.23^{bcd} ±0.3922	336	3.02^{e} ±0.7211
192	1.2725^{bcd} ±0.4029	360	1.5975^{d} ±0.4158

TABLE III
ANOVA RESULTS BY FACTORIAL TEST

Source	df	Mean Square	F	Sig.
Time	13	2.170	9.947	0.000**
Treatment	1	10.664	48.885	0.000**
Time * Treatment	13	0.360	1.652	$0.129^{n.s}$
Error	28	0.218		
Corrected Total	55			

n.s: Non-significant

**: Significant difference at the level of 0.001

C. FT-IR spectroscopy

Figure 3-a shows the FT-IR spectrum of the pure cellulose. The bands at 1664 and 1431 cm^{-1} indicate that carboxylic acid groups and carboxylate groups exist on the surface. The band of 2999 cm^{-1} is attributed to CH_2 stretching. The band at 1058 cm^{-1} could be associated with ether C-O-C functionalities. The band at 3415 cm^{-1} is attributed to the presence of hydroxyl groups (-OH). Comparison of the FT-IR spectrum of cellulose (3-a) with BC (3-b) indicated appropriate coincidence which

proved that the component produced by *G. xylinus* was cellulose.

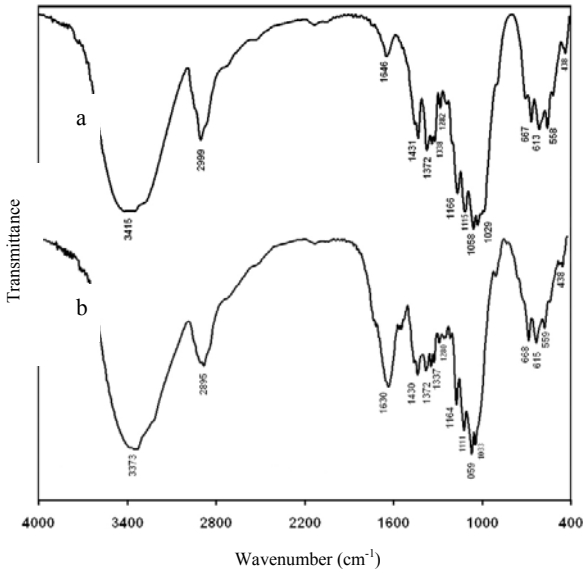

Fig. 3 FT-IR spectrum of (a) pure cellulose, (b) bacterial cellulose

D. SEM

Figure 4 shows the morphological structures of BC and cellulose fibers from lyophilized samples. According to the SEM images of samples, the diameter ratio of BC to cellulose that was represented in 4-b and 4-c figures was approximately 1/30 (412nm/12µm) which showed more delicacy of BC fibers relative to cellulose. This ratio may be even less 1/100 between the other fibers. More delicate BC fibers could influence its properties such as water-holding capacity, thermal stability and mechanical strength. The bacterial cellulose has a higher tensile strength, moldability and water holding capacity (up to 700 times its dry weight). Figures 4-a and 4-d at 1K magnification show more kink form of BC compared to cellulose.

E. X-Ray analysis

Figure 5 Shows XRD patterns of the samples. The area under XRD patterns is the measure of samples crystallinity which was 60.73% and 83.61% for BC and cellulose, respectively. Less crystallinity of BC makes it chemically a reactive component when participates in a chemical reaction. The pattern for BC exhibited two main peaks at 14.32° and 22.57°, corresponding to the 101 and 002 reflections, respectively. Cellulose crystallites showed main peaks at 14.59° and 22.92°, corresponding to the crystallographic peaks plane of 101 and 002, respectively. These data indicated that the BC and cellulose samples were the typical crystalline forms of cellulose I. The interplanar distances of the crystallites (d-spacings) could be calculated with Bragg's law (equation 1).

$$n\lambda = 2d \sin\theta \quad (1)$$

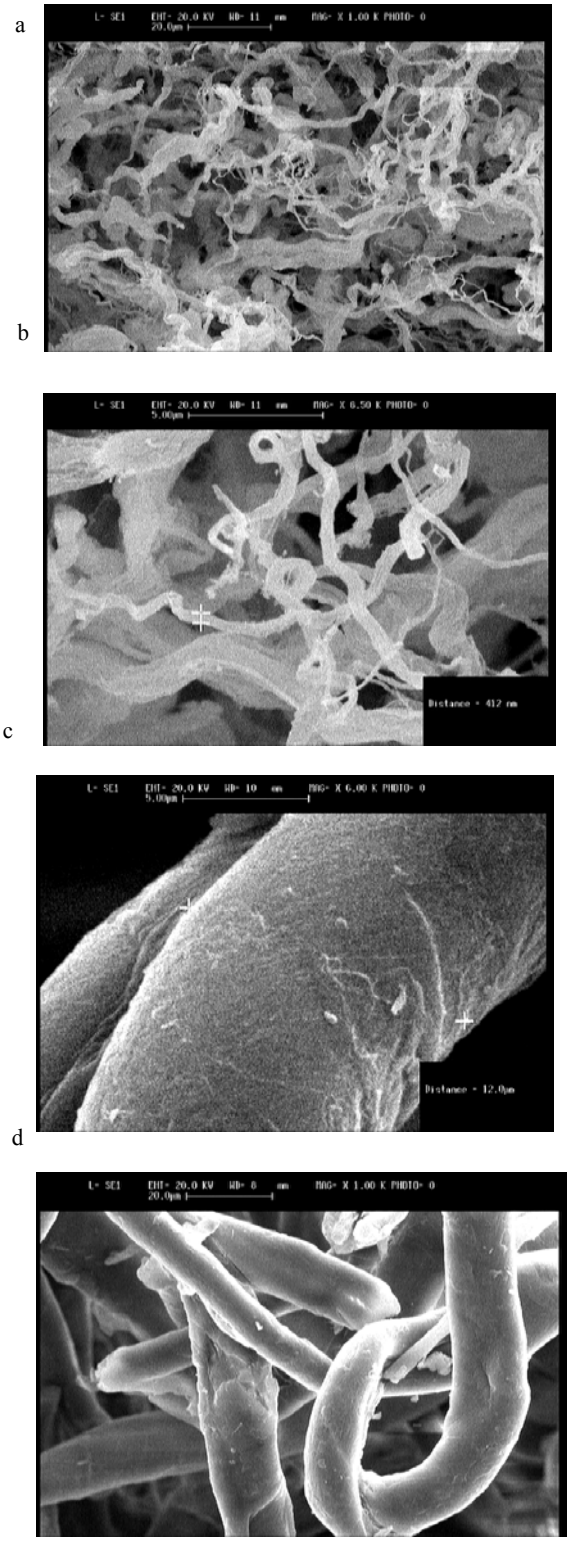

Fig. 4 SEM images of (a,b) BC, (c,d) standard cellulose

Where n is an integer determined by the order given, λ is the wavelength of the X-rays (and moving electrons, protons and neutrons), d is the spacing between the planes in the atomic lattice, and θ is the angle between the incident ray and the scattering planes. The d-spacings of 101 and 002 planes for BC were 6.22 and 3.93 A˚, and for cellulose were 6.06 and 3.89A˚, respectively.

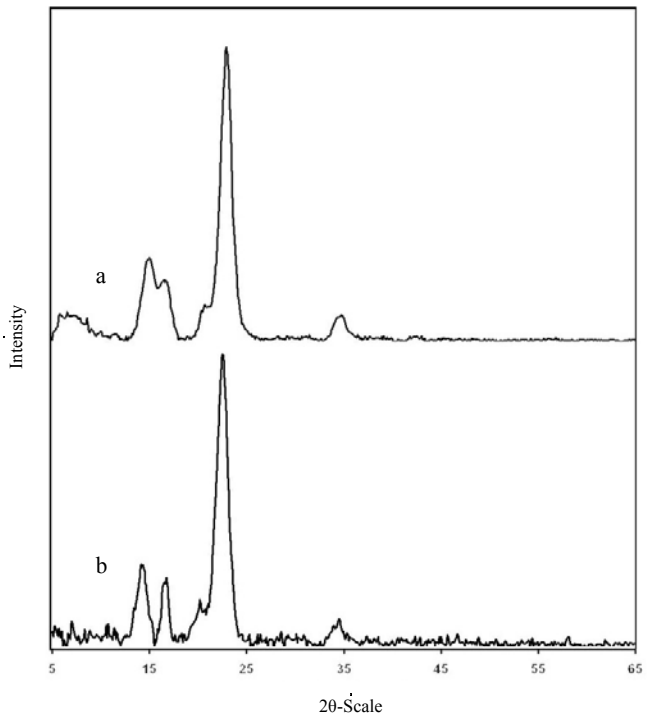

Fig. 5 XRD graphs of (a) cellulose, (b) BC.

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