Carbohydrate Polymers 86 (2011) 1187–1191

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

# Bacterial synthesized cellulose nanofibers; Effects of growth times and culture mediums on the structural characteristics

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ARTICLE INFO

*Article history:*
Received 9 May 2011
Received in revised form 27 May 2011
Accepted 6 June 2011
Available online 12 June 2011

*Key words:*
Bacterial cellulose
Nanofibers
*Gluconacetobacter xylinus*
FT-IR spectroscopy
X-ray diffraction

ABSTRACT

In this study the effects of growth times and culture mediums on the structural characteristics of bacterial cellulose have been investigated. Bacterial cellulose (BC) nanofibers were synthesized by *Gluconacetobacter xylinus*. BC pellicles were compared using SEM, FT-IR and X-ray diffraction techniques. The crystallinity index (CrI) and crystallite size (CrS) were calculated based on X-ray measurements. Three growth times (7, 14 and 21 days) and three culture mediums (A, B and C) were applied. SEM micrographs showed that increasing growth time up to 7 days improves the microfibril branches crossing to each other and the number of bundles. However, further increase in growth time (21 days) results in decrease in the microfibril network. On the other hand the hydrogen and C–H bonds were developed by the increase in growth time. In conclusion, BC synthesized in medium B for 7 days had superior properties in terms of CrI, CrS and microfibril networks.



## 1. Introduction

Cellulose, the most abundant biopolymer in nature, can be synthesized by plants, some animals and a large number of microorganisms (Castro et al., 2011; Czaja, Romanovicz, & Malcolm Brown, 2004). It is composed of glucose monomers connected by β(1–4) glycosidic linkages, and its chemical formula is $(C_6H_{10}O_5)_n$. Cellulose forms the basic structural matrix of cell walls in almost all plants, in many fungi, and in some types of algae. However, this form of cellulose contains many impurities, including hemicellulose and lignin, and therefore, harsh chemical treatments are required to remove these impurities. Chemical treatments result in irreversible changes in cellulose structure, which permanently strip the polymer of its useful characteristics. Plant-derived cellulose has been important in the production of paper and wood-based products. However, the increasing demand for natural cellulose and thus increased consumption of wood as raw material of cellulose, are causing deforestation and creating global environmental issues (Park, Park, & Jung, 2003).

In recent years, a great deal of interest has been created worldwide on the production of cellulose by using a new process (biotechnology technique) that reduces the environmental impact to a minimum. Studies suggest that bacterial cellulose or biocellulose (BC) may be a better choice for manufacturing cellulose products (Castro et al., 2011; Keshk, Razek, & Sameshima, 2006; Nakagaito, Nogi, & Yano, 2010; Pourramezan, Roayaei, & Qezelbash, 2009). It has a chemical structure similar to the cellulose, where hydroxyl functional groups exist (Fig. 1).

Bacterial cellulose, an exopolysaccharide, is produced by many species of bacteria, such as those in the genera of *Acetobacter*, *Agrobacterium*, *Achromobacter*, *Aerobacter*, *Azotobacter*, *Sarcina ventriculi*, *Salmonella*, *Escherichia* and *Rhizobium* (Moosavi-Nasab & Yousefi, 2011; Sani & Dahman, 2010). The structural features of bacterial cellulose are influenced by the kind of bacterial strain (Table 1). Among the mentioned genera, *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) is one of the most commonly used/studied sources of bacterial cellulose (Keshk et al., 2006; Nguyen, Gidley, & Dykes, 2008). It was also reported as the most efficient producer (El-Saied, Basta, & Gobran, 2004). This is a gram-negative bacterium, strictly aerobic, capable of producing cellulose extracellularly at temperatures between 25 and 30 °C and pH from 3 to 7, using glucose, fructose, sucrose, mannitol, among others, as carbon sources (Castro et al., 2011).

In terms of chemical structure, bacterial cellulose is identical to that produced by plants. However, it exhibits higher crystallinity (Nakagaito et al., 2010), water-holding capacity (Saibuatong & Phisalaphong, 2010), degree of polymerization (Dahman, Jayasuriya, & Kalis, 2010), and mechanical strength and

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doi:10.1016/j.carbpol.2011.06.011

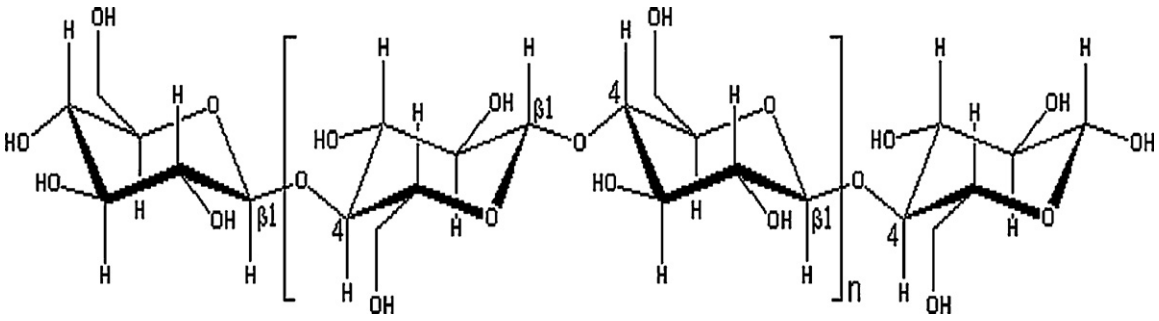

**Fig. 1.** Cellulose structure.

purity (Castro et al., 2011). It contains no lignin, hemicellulose or other natural components. These properties make it an interesting biomaterial for applications as nutritional component (Sani & Dahman, 2010), artificial skin (Hungund & Gupta, 2010), composite reinforcement (Czaja et al., 2004), electronic paper (Jonas & Farah, 1998), flexible display screens (Nakagaito et al., 2010), and in traditional applications where plant cellulose is used (Castro et al., 2011). In recent years, an interest has developed in producing bacterial cellulose on a large commercial scale (Castro et al., 2011; Czaja et al., 2004). However, bacterial cellulose is still expensive compared with other popular commercial organic products, therefore, its use is limited. It is important to develop methods to produce bacterial cellulose at the lowest cost possible. A significant point that many researchers have investigated is the culture conditions and their effects.

This work aimed at studying the effects of various growth times and culture mediums on the morphology and structural characteristics of bacterial cellulose. The cellulose nanofibers were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transform infrared (FT-IR) spectroscopy.

## 2. Materials and method

### 2.1. Materials

The organism used was *G. xylinus* (BPR 2004), which was obtained from the DMSZ Laboratory, Germany. Bacteria were resuscitated by incubation on GYC (20 g glucose, 2 g yeast extract, 4 g $CaCO_3$, 3 g agar, and 200 $cm^3$ $dH_2O$) at 26 °C for 2 days. Working cultures were routinely prepared on GYC and stored at 4 °C until use.

Three different growth culture mediums were used in this study. Table 2 presents the chemical compositions of the used mediums.

**Table 1**
Effect of microorganisms on cellulose producers.

| Organisms (genus) | Cellulose produced | Biological role |
|---|---|---|
| *Acetobacter* | Extracellular pellicle | To keep in aerobic |
| *Acetobacter* | Cellulose ribbons | Environment |
| *Achromobacter* | Cellulose fibrils | Flocculation in wastewater |
| *Aerobacter* | Cellulose fibrils | Flocculation in wastewater |
| *Agrobacterium* | Short fibrils | Attach of plant tissues |
| *Alcaligenes* | Cellulose fibrils | Flocculation in wastewater |
| *Pseudomonas* | No distinct fibrils | Flocculation in wastewater |
| *Rhizobium* | Short fibrils | Attached to most plants |
| *Sarcina* | Amorphous cellulose | Unknown |
| *Zoogloea* | Not well defined | Flocculation in wastewater |

Source: El-Saied et al. (2004).

### 2.2. Culture conditions

The culture medium (A) was used to study the effect of culture time (7, 14 and 21 days) on cellulose structure. The preparation procedures were reported elsewhere (Nguyen et al., 2008). The produced bacterial cellulose pellicles were purified by soaking in ionized water at 90 °C for 2 h and boiled in a 0.5 M NaOH solution for 15 min. Consequently, pellicles were washed with ionized water for several times and soaked in 1 wt.% NaOH for 2 days. Finally, the pellicles were stored in ionized water to pH 7 at room temperature prior to test.

### 2.3. Scanning electron microscopy (SEM)

SEM was used to observe the microorganism morphology and its distribution in the membrane. A CamScan model MV2300 scanning electron microscope operating at 15 kV was used. The samples were dried and covered with a 1 nm gold layer on copper supports.

### 2.4. Fourier transform infrared (FT-IR) spectroscopy

Each cellulose sample was air-dried on a glass slide in the form of a thin film, and then placed across a hole in a magnetic holder. FT-IR spectroscopy was used primarily to identify the chemical structure of the membrane. The IR spectra of membranes were measured at wave numbers ranging from 4000 to 400 $cm^{-1}$ using a FT-IR spectrum RXI.

### 2.5. X-ray diffraction

Dried films of cellulose microfibrils were X-rayed using Bruker Axe (Model D8 Discover, Kalsrush, Germany) equipment. X-ray diffraction patterns were recorded at the $CuK_\alpha$ radiation wavelength ($\lambda$ = 1.54 Å), generated at a voltage of 40 kV and a filament emission of 30 mA. Samples were scanned from 2° to 50° 2$\theta$-range at scan speed of 0.5°/min.

The crystallinity index (CrI) and crystallite size (CrS) were calculated based on X-ray diffraction measurements. The CrI was calculated from the ratio of the height of the 0 0 2 peak ($I_{002}$, $2\theta$ = 22.6°) and the height of minimum ($I_{am}$) between the 0 0 2

**Table 2**
Chemical compositions of used mediums.

| Medium | Compositions |
|---|---|
| A | 20 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L $Na_2HPO_4$, 1.15 g/L citric acid·$H_2O$ |
| B | 20 g/L manitol, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L $Na_2HPO_4$, 1.15 g/L citric acid·$H_2O$ |
| C | 15 g/L glucose, 2.5 g/L poly peptone, 2.5 g/L yeast extract, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 500 $cm^3$ $dH_2O$ |

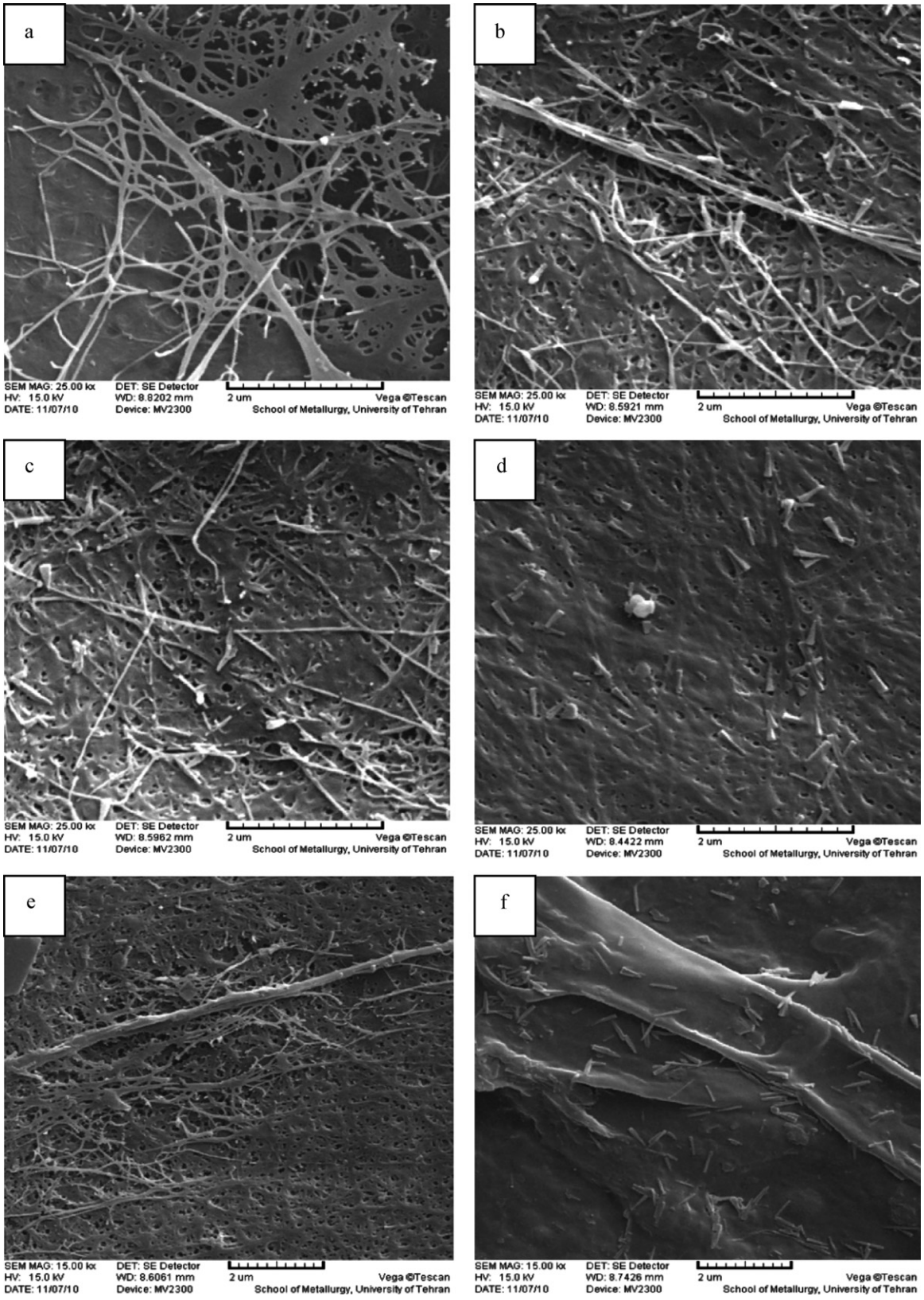


**Fig. 2.** SEM images of bacterial cellulose synthesized after 7 days (a), 14 days (b), 21 days (c), and in medium A (d), B (e) and C (f).

and the 1 1 0 peaks ($I_{am}$, $2\theta = 18^{\circ}$) (Thygesen, Oddershede, Lilholt, Thomsen, & Ståhl, 2005).

$$\mathrm{CrI} = \frac{100(I_{002} - I_{am})}{I_{002}} \quad (1)$$

The CrS was estimated using Scherrer equation as following:

$$\mathrm{CrS} = \frac{K\lambda}{\beta \cos\theta} \quad (2)$$

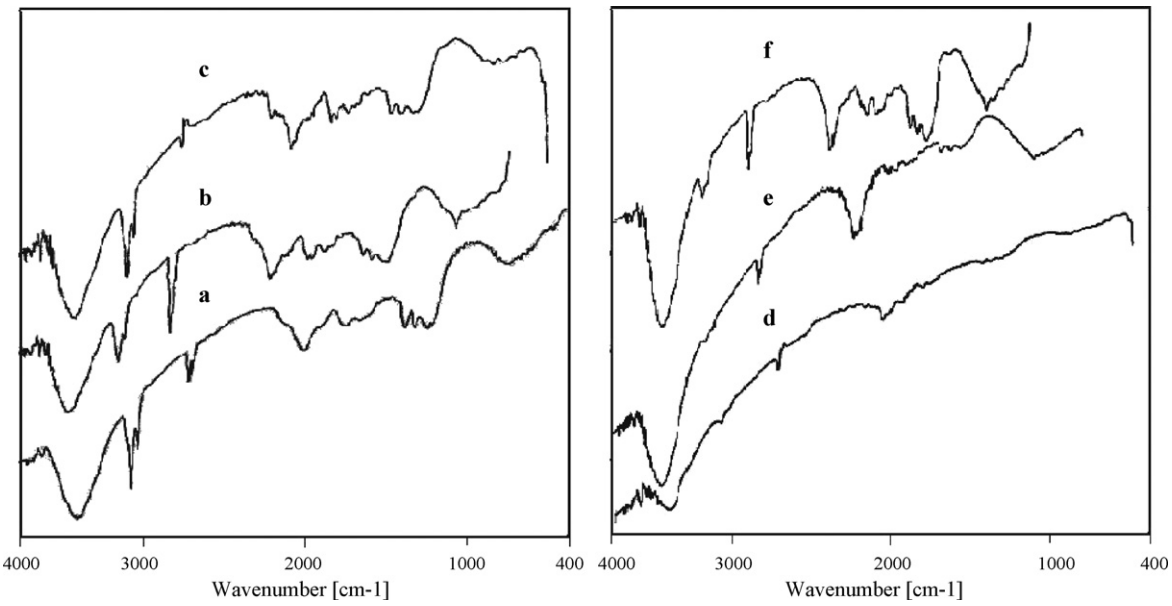


**Fig. 3.** FT-IR spectra of bacterial cellulose synthesized after 7 days (a), 14 days (b), 21 days (c), and in medium A (d), B (e) and C (f).

where $k$ is the shape factor (0.9), $\lambda$ is the X-ray wavelength (1.54 Å), $\beta$ is the line broadening at half the maximum intensity (FWHM) in radians, and $\theta$ is the Bragg's angle.

## 3. Results and discussion

### 3.1. SEM

The surface pellicle formed by *G. xylinus* sp. was examined using SEM. It is notable that the bacterial cellulose pellicles, which were synthesized in the culture mediums A and B, were stable, unlike those in medium C that were unstable. A close observation revealed that mostly uniaxially oriented ribbons characterize cellulose were formed in culture mediums A and B. The micrograph in Fig. 2a–c reveals that the rod-shape of microfibrils bundles. This compact cellulose network structure is made up a random assembly of fibrils.

A time course of cellulose synthesis shown in Fig. 2a–c indicates that after 14 days of culture the branches crossed to each other and bundles were increased. However, after 14 days no further significant increase in cellulose synthesis was observed. The formation of bundles is due to the increasing hydrogen bonds. The ramifications of bacterial cellulose are seen clearly at images of mediums A and B, while no such ramification is seen in medium C (Fig. 2d–f).

### 3.2. FT-IR spectroscopy

Fourier transform infrared (FT-IR) spectroscopy of bacterial cellulose films was carried out in order to detect the effect of growth time and medium on shift of peaks. The FT-IR spectra of all samples were shown in Fig. 3. The region 3600–3200 $cm^{-1}$ is assigned to hydroxyl functional groups and hydrogen bonds (Barud et al., 2008). Effect of growth time can be explained by peak intensity increase from 7 to 21 days (Fig. 3a–b). The intensity of peak in this region of synthesized bacteria cellulose in medium B is more intensive than the other two mediums (Fig. 3d–f). The absorption bonds at 2900–2820 $cm^{-1}$ were assigned to C–H stretching. Corresponding to region 3600–3200 $cm^{-1}$, in this spectrum, also increasing the duration of growth resulted in increase in intensity of peak. The C–H bond was enhanced in medium B compared with the other two mediums. The main bonds in all samples can be assigned to: 3500 $cm^{-1}$ (OH stretching), 3246 $cm^{-1}$ (H-bond), 2908 $cm^{-1}$ (CH stretching of $CH_2$ and $CH_3$ groups), 2700 $cm^{-1}$ ($CH_2$), 1654 $cm^{-1}$ (water OH bending), 1435 $cm^{-1}$ ($CH_2$ symmetric bending), 1370 $cm^{-1}$ (CH bending), 1160 $cm^{-1}$ (anti symmetric bridge COC stretching), 1111 $cm^{-1}$ and 1056 $cm^{-1}$ (anti symmetric out-of-phase bending), and 667 $cm^{-1}$ and 619 $cm^{-1}$ (OH out-of-phase bending).

### 3.3. XRD

Two common crystalline forms of cellulose, designated as I and II, are distinguishable by X-ray diffraction. It is known that in the meta stable cellulose I, which is synthesized by the majority of plants and also by *G. xylinus* in static culture, parallel β-1,4 glucan chains are arranged uniaxiallry, whereas β-1,4 glucan chains of cellulose II are arranged in random manner. They are mostly nonparallel and linked with a larger number of hydrogen bonds, which results in higher thermodynamic stability of the cellulose II (Yu & Atalla, 1996). Detailed study of structural characteristics carried out using electron diffraction analyses (Sugiyama, Persson, & Chanzy, 1991) and (CPMAS) $^{13}C$ NMR (Yamamoto & Horii, 1993) revealed that native cellulose is a composite of two different crystalline phases called $I_\alpha$ and $I_\beta$. Normally, *G. xylinus* cellulose displays characteristics of highly crystalline, $I_\alpha$-rich cellulose (VanderHart & Atalla, 1984). Fig. 4 shows XRD patterns for bacterial cellulose synthesized with various growth times and culture mediums. Diffraction peaks at 14.5° and 22.6° are assigned to the cellulose $I_\alpha$ and $I_\beta$ phases ($1\,0\,0\,1_\alpha$, $1\,1\,0\,1_\beta$ and $0\,1\,0\,1_\beta$ planes at 14.5° and $1\,1\,0\,1_\alpha$ and $2\,0\,0\,1_\beta$ at 22.6°) (Maeda, Nakajima, Hagiwara, Sawaguchi, & Yano, 2006).

The results of the crystallite size and crystallinity index are presented in Table 3. As it can be seen, both the crystallite size and

**Table 3**
Crystallinity index (CrI) and crystallite size (CrS) of bacterial cellulose samples.

| Sample types | CrI (%) | CrS (nm) |
|---|---|---|
| BC-7 | 80.6 | 12.7 |
| BC-14 | 77.0 | 7.6 |
| BC-21 | 75.7 | 6.2 |
| BC-A | 77.0 | 7.6 |
| BC-B | 78.3 | 9.8 |
| BC-C | 62.8 | 5.6 |

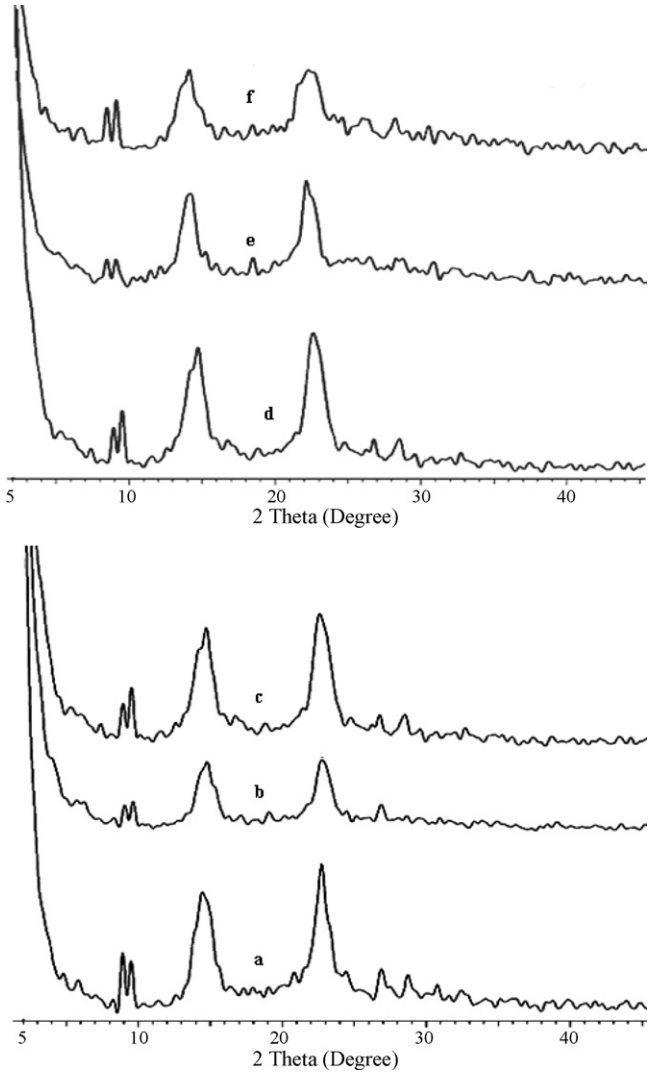


**Fig. 4.** X-ray diffraction patterns of bacterial cellulose samples synthesized after 7 days (a), 14 days (b), 21 days (c), and in medium A (d), B (e) and C (f).

crystallinity index decreased with passing of time (Fig. 4a–c). In addition, the crystallite size and crystallinity index was more in bacterial cellulose, formed in medium B compared with the other two mediums (Fig. 4d–f).

## 4. Conclusions

Based on the results of this study the following conclusions can be drawn.

- Increasing the growth time improved the number of microfibril branches crossing to each other and the numbers of bundles formed. However, after 14 days, no further significant increase in the cellulose formed was observed.
- Hydrogen and C–H bonds increased with increasing growth time in bacterial cellulose samples.
- The CrI and CrS decreased with increasing growth time.
- The results revealed that bacterial cellulose synthesized in medium B for 7 days had superior properties in terms of CrI, CrS and microfibril networks compared to the other used mediums.

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