Biotechnological production of cellulose by *Gluconacetobacter xylinus* from agricultural waste

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Abstract
The purpose of this study was to utilize low quality date syrup, a rich and available source of nutrient in Iran, for the production of bacterial cellulose using *Gluconacetobacter xylinus*. Static batch fermentation for the purpose of cellulose production by *G. xylinus* (PTCC, 1734) was studied using low quality date syrup and sucrose solution (Bx. 10%) as fermentation media at 28°C. Results showed that maximum yields of bacterial cellulose after 336 h fermentation were 4.35 and 1.69 g/100 ml of date syrup and sucrose media, respectively. The FT-IR spectrum of commercial plant cellulose as a standard was similar to that of bacterial cellulose. To determine the physical structure of the bacterial cellulose and standard cellulose fibers, scanning electron microscopy (SEM) was carried out. The results revealed more delicacy in structure of bacterial cellulose. Determination of crystallinity of the samples using X-ray diffractometry demonstrated that the crystallinity level of standard cellulose (83.61%) was more than that of bacterial cellulose (60.73%). This study obviously showed the ability of low quality date syrup, a suitable and cheap carbon source, to be used as a substrate in a fermentation medium for production of cellulose by *Gluconacetobacter xylinus.*
Keywords**:** Cellulose; Date syrup; *G. xylinus*; Fourier transform infrared spectroscopy; Scanning electron microscopy; X-ray diffractometry

INTRODUCTION

Date production is an international agricultural industry which has produced approximately 5.4 million metric tons (Mt) of fruit over the period 1999-2001. The date fruit is produced largely in the hot arid regions of Southwest Asia and North Africa. On average, Iran, Saudi Arabia and Iraq had almost half of the harvested area of the world during this period. Date syrup (DS) was approved to contain adequate nutrients in satisfactory amounts to be suitable for growth of microorganisms (Khiyami *et al*., 2008). Date syrup is a viscous liquid produced as a by-product of the date industry; it is rich in carbohydrates (75% w/w) and contains small amounts of protein and fat (1.1 and 2.9%, respectively) (Al-Farsi *et al*., 2007) in addition to many macro- and micro-elements (Al-Hooti *et al*., 2002).

Exopolysaccharides are long chain polysaccharides which are composed of branched, repeating units of sugars or sugar derivatives, such as glucose, galactose and rhamnose in different amounts. They are classified into two groups: homopolysaccharides (cellulose, dextran, mutan, pullulan, curdlan), and heteropolysaccharides (gellan and xanthan) (Chawla *et al*., 2009). Cellulose is the most abundant macromolecule on earth (Zhao *et al*., 2007) that is mostly produced by vascular plants. Cellulose is an unbranched polymer of β-1→4-linked glucopyranose residues. A substitute to reduce the demand from plants is the production of cellulose from another resource, such as the use of a microbial system (Brown, 2004). Microbial cellulose is an exopolysaccharide produced by various species of bacteria, such as those of the genera *Gluconacetobacter* (formerly *Acetobacter*), *Agrobacterium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, and *Salmonella* (Chawla *et al*., 2009). *Gluconacetobacter xylinus*, is one of the best bacterial species for the production of bacterial cellulose in large-scale and utilizes a wide

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variety of substrates. Bacterial cellulose (BC) is chemically pure, free of undesirable components such as lignin and hemicellulose (there is no need for chlorine chemical bleaching) and has high polymer crystallinity and high degree of polymerization that distinguishes it from other forms of cellulose (Khan *et al*., 2007). The biochemical pathway that leads to the production of bacterial cellulose is shown in Figure 1. BC has a higher crystallinity, tensile strength, moldability and water holding capacity (up to 700 times its dry weight) (Bielecki *et al*., 2002). Its strength and toughness makes it appropriate to use instead of common cellulose in many cases that it is unsuited (Bielecki *et al*., 2002).

Because of its unique properties and structure, bacterial cellulose has important applications in a variety of food formulations (Khan *et al*., 2007). It is especially used when low-level use, lack of flavor interactions, foam stabilization, and stability over a wide range of pH, temperature, and freeze-thaw conditions are required. Potential uses include pourable and spoonable dressings, sauces, and gravies; frostings and icings; sour cream and cultured dairy products; whipped toppings and aerated desserts, and frozen dairy products (Chawla *et al*., 2009; Khan *et al*., 2007). The bacterial cellulose in combination with other agents like sucrose and carboxymethyl cellulose improve the dispersion of the product. Besides the above, its potential food applications also include a low-calorie additive, thickener, stabilizer, texture modifier, pasty condiments, and ice cream (Khan *et al*., 2007).

In recent years, various carbon sources including monosaccharides, oligosaccharides, alcohols, sugar alcohols and organic acids, have been used to maximize bacterial cellulose production by various *G. xylinus* strains (Ishihara *et al*., 2002; Keshk *et al*., 2006). Based on the unique functional properties of the bacte-

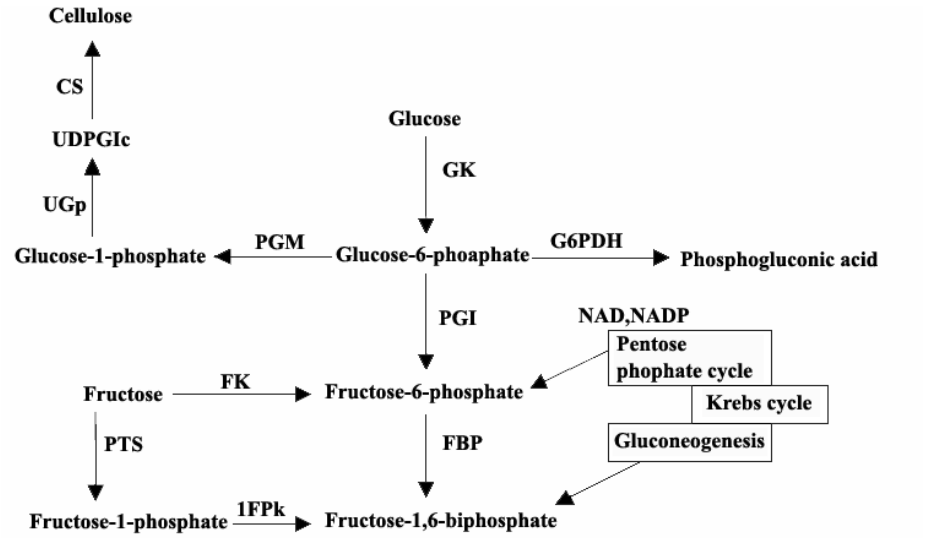

Figure 1. Biochemical pathway for cellulose synthesis by *G. xylinum*. (CS cellulose synthase, GK glucokinase, FBP fructose-1,6-biphosphate phosphatase, FK fructokinase, 1FPk fructose-1-phosphate kinase, PGI phosphoglucoisomerase, PMG phosphoglucomutase, PTS system of phosphotransferases, UGP pyrophosphorylase uridine diphosphoglucose, UDPGlc uridine diphosphoglucose, G6PDH glucose-6-phosphate dehydrogenase, NAD nicotinamide adenine dinucleotide, NADP nicotinamide adenine dinucleotide phosphate) (Chawla *et al*., 2009).

Table 1. Chemical characteristics of the prepared date syrup.

Component	Quantity (%)	Reference
Total sugar	73.1	AOAC (Lane-Eynons method), 1995
Reducing sugar	67.3	AOAC (Lane-Eynons method), 1995
Ash	1.52	AOAC, 1995
Moisture content	24.15	AOAC, 1995
Protein	1.3	AOAC, 1995 (Biuret test)

rial cellulose that were mentioned above, in this study, bacterial cellulose was produced by *G. xylinus* from an agricultural waste (low quality date drup) which is used as a cheap and available substrate in Iran. In addition sucrose fermentation medium was used for comparison of production yields. The chemical structure of the produced bacterial cellulose was characterized by Fourier transform infrared spectroscopy (FT-IR) and X-ray diffractometry. The distinctiveness of physical structure of bacterial cellulose fiber, determined using scanning electronic microscopy (SEM), was compared to that for standard cellulose.

MATERIALS AND METHODS

Preparation of date syrup: The initial extract was prepared by soaking 200 g of stone free, low quality date fruits in 500 ml of distilled water, then mixed in a blender for 1 min at low speed, and for an additional 3 min at a higher 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 make a 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. The results of chemical analysis of the prepared date syrup are shown in Table 1.

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 test tubes containing tomato serum medium and were subcultured monthly. The typical composition of the stock culture medium was 50 g/l of glucose, 5 g/l of bactopeptone, 5 g/l of yeast extract and 10% by volume tomato juice (pH 6.8). The stock cultures were stored at 5ºC to slow down the growth and cellulose production.

Inoculum preparation: A culture medium compose of 5% glucose, 0.5% bactopeptone, 0.5% yeast extract, 0.27% monobasic sodium phosphate 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 min 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 28ºC. The cells were collected by centrifugation (2500 g, 20 min) and resuspended in liquid medium and the bacteria were inoculated into liquid fermentation medium in Erlenmeyer flasks containing date syrup or sucrose.

Cellulose production: In this study, *G. xylinus* was grown in a generic medium derived by Schramm and Hestrin (1954), the composition of which is shown in Table 2. After preparation of inocula, bacterial cells were inoculated into liquid fermentation media in Erlenmeyer flasks containing date syrup or sucrose (Bx. 10) as a carbon source. Fermentation was performed in a static state (set at 28ºC with a medium pH of 6.8). Samples were taken from the fermentation media after 48 h, at 24 h intervals. 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.

Yield of bacterial 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 produced bacterial cellulose 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 sodium hydroxide base was removed. Dry weight was measured after drying the film for three days at 45ºC. The dried bacterial cellulose samples were then weighed, and values were reported as mg/100 ml of the original medium.

Pellicle producion and purification: Bacterial cellulose was produced by *G. xylinus* in a 2 l Erlenmeyer flask at 28ºC for 30 days. After incubation, the pellicle produced on the surface of the media containing date syrup was separated. For removal of the microbial

Table 2. The composition of the culture medium used for seeding and fermentation.

Components	Quantity
Carbon source (Bx. 10)	1 L
Na_2HPO_4	2.7 g
Bactopeptone	5 g
Yeast extract	5 g
Citric acid	1.5 g

cells, the pellicles were washed with water, 4% (w/v) sodium hydroxide solution in a boiling bath (30 min), 6% (v/v) acetic acid, and then water, successively.

Fourier transform infrared spectroscopy: Standard sample (Merck, Germany) and the bacterial cellulose were mixed well 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 in the range of 4000-400/cm.

Scanning electron microscopy: 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 (SEM) (model 5526, Cambridge, UK) at 20 kV.

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 degrees per min from 5-60° 2θ. The samples were lyophilized overnight using a lab-scale freeze dryer and pressed into a thin and flat layer (~1.5 mm thickness) for analysis.

Statistical data analysis: One-way analysis of variance (ANOVA) by SPSS 17 software was performed for the analysis of the yield data. Duncan test at 95% confidence level was accomplished to determine the effect of different fermentation times on yield of production. Factorial test was carried out to indicate simultaneous effects of treatments (production in different carbon sources) and fermentation times. The independent T-test at 95% confidence level was used to compare the yield in sucrose with that of the date syrup medium.

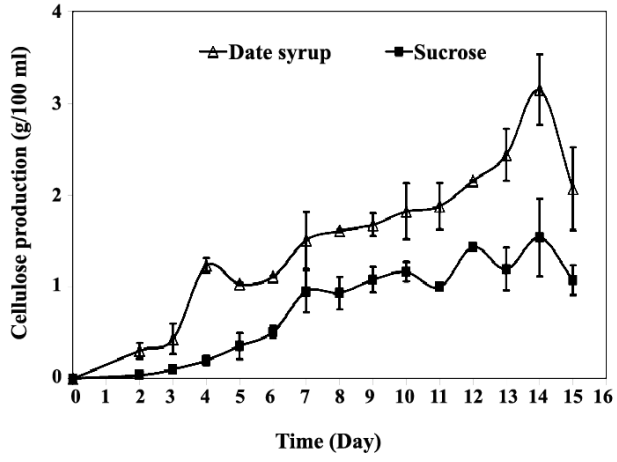

Figure 2. Yield of bacterial cellulose production in fermentation media containing both date syrup and sucrose during fermentation.

RESULTS

Yield of bacterial cellulose production: The rate of BC production in fermentation media containing date syrup was monitored over a period of 360 h of incubation at 28ºC in a static state (Fig. 2). According to the results, the production of BC in the fermentation medium containing sucrose remained relatively constant but when the fermentation was carried out in the presence of date syrup, BC production continuously increased until the 14th day of the fermentation period. *G. xylinus* grown in fermentation medium containing date syrup gave a significantly ($p<0.001$) higher yield of BC than fermentation medium containing sucrose. The highest yield of production was obtained after 14 days of incubation at 28ºC for both date syrup and sucrose fermentation media. Efficient cellulose production by this bacterium lies in its ability to synthesize glucose from various carbon substrates, followed by glucose polymerization to cellulose. *G. xylinus* has two main operative amphibolic pathways: the pentose phosphate cycle for the oxidation of carbohydrates and the Krebs cycle for the oxidation of organic acids and related compounds (Ross *et al*., 1991). As the results show, yields of cellulose in date syrup medium was approximately 2 times more than that for the sucrose medium. This is due to the adequate presence of the reducing sugars (67.3%), especially glucose which is the initial substrate for cellulose production (Fig. 1). The yields from each sampling time were tested by Duncan's multiple range tests. The obtained results

Table 3. Results of Duncan's test for yields of production.

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

showed four significant groups that are shown in Table 3. These results demonstrated that the yield after 336 h of fermentation was significantly different relative to all the other yields, at the level of 0.05. Factorial test results (Table 4) represented the significant difference for treatment and sampling times alone at the level of 0.001, while time treatments interaction did not show a significant difference, even at the level of 0.05. The T-test results demonstrated that there was a significant difference ($p<0.001$) related to the yields of production between the two fermentation media.

Table 4. ANOVA results using the 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	0.129n.s
Error	28	0.218		
Corrected Total	55			

n.s: Non-significant, **: Significant difference at level of 0.001.

Fourier transform infrared spectroscopy: Figure 3 shows the FT-IR spectra of the standard cellulose and BC, respectively. The bands at 1664 and 1431/cm indicate that carboxylic acid groups and carboxylate groups exist on the surface. The band at 2999/cm is attributed to CH_2 stretching. The band at 1058/cm could be associated with ether C-O-C functionalities. The band at 3415/cm is attributed to the presence of hydroxyl groups (-OH) (Goyanes *et al*., 2007). The FT-IR spectrum at the region of 3230-3455/cm indicates intermolecular and intramolecular hydrogen bonds (Oh *et al*., 2005). The intensity ratio of the band at 3240-3349/cm in BC spectrum was lower than that in standard cellulose spectrum (Fig. 4), which indicated that the intermolecular hydrogen bonds in BC were weaker relative to those in the standard cellulose (Sugiyama *et al*., 1991). Overall, comparison of the FT-IR spectrum of cellulose with that of BC demonstrated appropriate coincidence, which proved that the component produced by *G. xylinus* was cellulose.

Scanning electron microscopy: Figure 5 shows the morphological structures of BC and standard cellulose fibers obtained from lyophilized samples. SEM images of samples, showed more delicacy of BC fibers relative to cellulose. The diameter ratio of standard cellulose to BC is approximately 1/30 or less (Moosavi-Nasab and Yousefi, 2010; Jung *et al*., 2007). The higher delicacy of the BC fibers could influence its properties, such as water-holding capacity, thermal stability and mechanical strength. For instance bacterial cellulose has a higher tensile strength, moldability and water holding capacity (up to 700 times its dry weight) (Jung *et al*., 2005). SEM images of standard cellulose

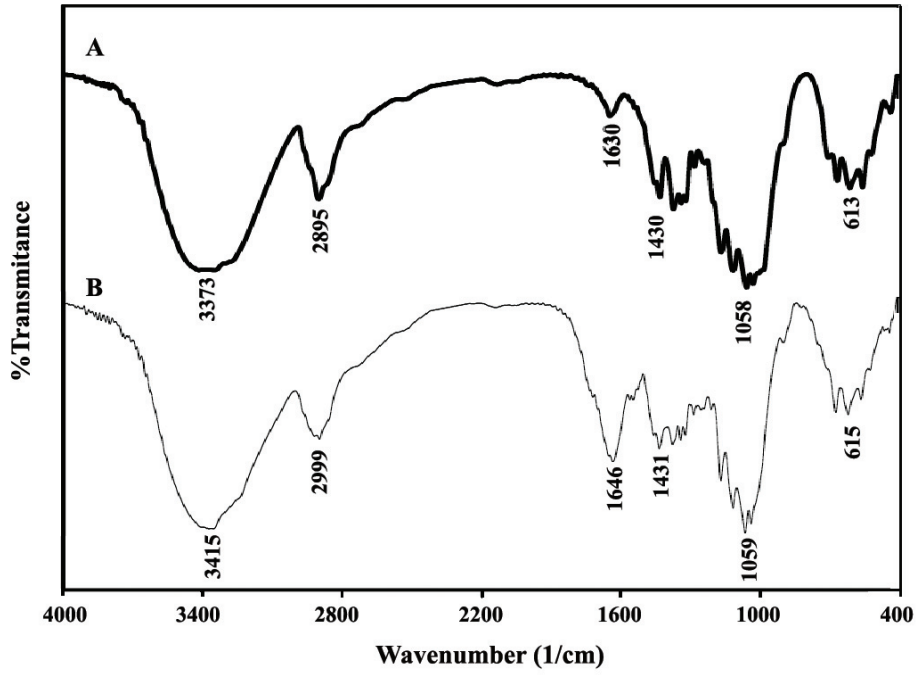

Figure 3. FT-IR spectrum of A: standard cellulose and B: BC.

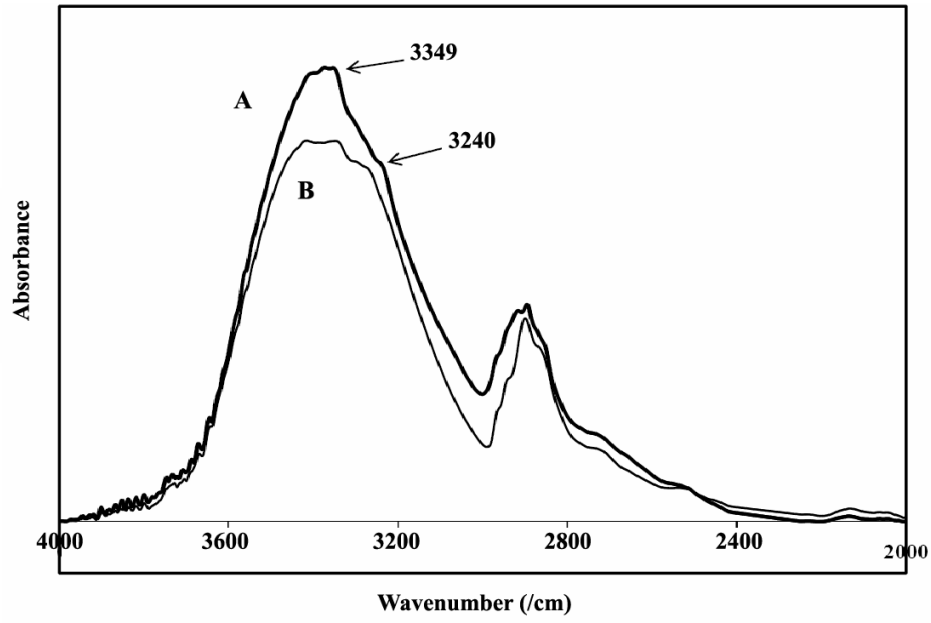

Figure 4. FT-IR spectra of 4000-2000/cm of A: BC and B: standard cellulose.

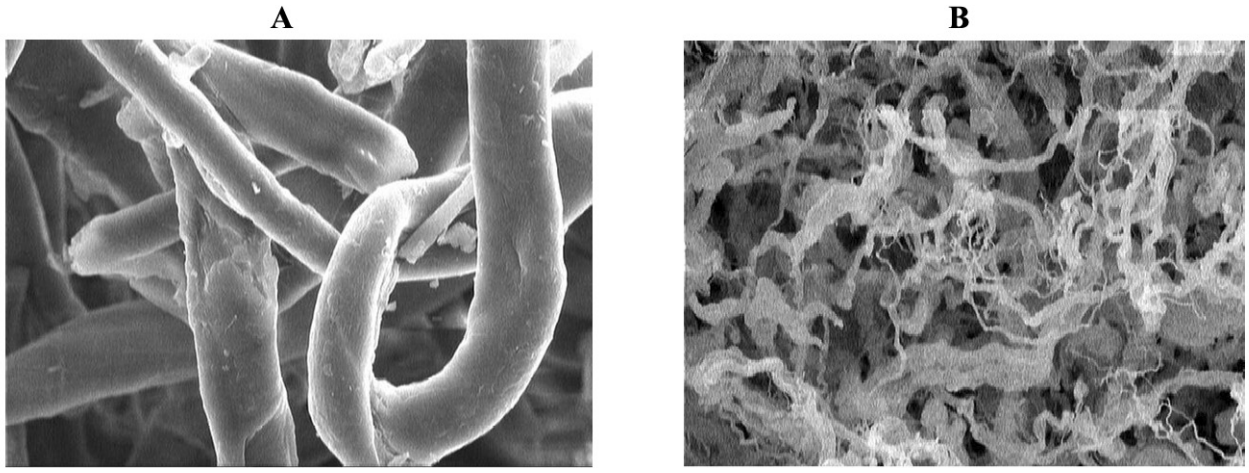

Figure 5. SEM images of A: standard cellulose and B: BC.

and BC (Fig. 5) at 1K magnification obviously showed the more kinked form of BC when compared to the standard cellulose.

X-Ray analysis: Figure 6 shows that XRD patterns of the samples. The area under the XRD patterns is the measure of the sample's crystallinity, which was 60.73% and 83.61% for BC and cellulose, respectively. The pattern for BC exhibits two main peaks at 14.32 and 22.57º, corresponding to the (101) and (002) reflections, respectively. Cellulose crystallites show main peaks at 14.59 and 22.92º, corresponding to the crystallographic peaks planes of (101) and (002), respectively. These data indicated that the BC and cellulose samples were the typical crystalline forms of cellulose I (Yan *et al*., 2008). The interplanar distances of the crystallites (d-spacings) could be calculated with Bragg's law (equation 1).

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

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

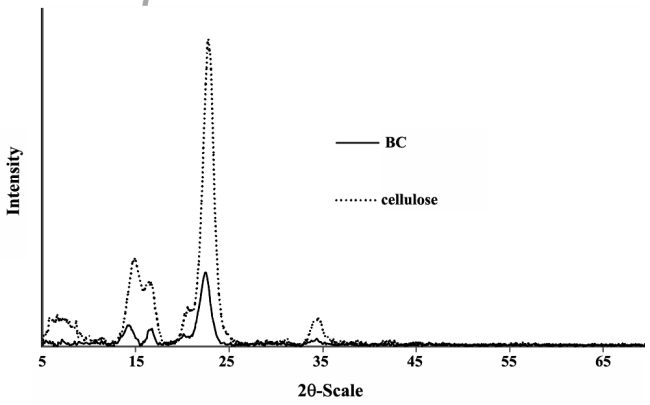

Figure 6. XRD patterns of standard cellulose and BC.

planes in the atomic lattice, and θ is the angle between the incident ray and the scattering planes (Yan *et al*., 2008). The d-spacings of (101) and (002) planes for BC were 6.22 and 3.93 Aº, respectively, while those for cellulose were 6.06 and 3.89Aº, respectively.

DISCUSSION

As mentioned before, Iran is one of the biggest producers of the date fruit in the world. High percentage of the date fruit in Iran are of low quality hindering export and local consumption thus, it seems necessary to use this source of energy for production of value-added components such as bacterial cellulose. Date syrup is an attractive by-product of dates which can be substituted in different food formulations. It has potential to be used as a substrate replacing common carbon and mineral sources in single cell protein (the protein utilizable as food or feed) production. It also contains a substantial level of nutrients that are required for the growth of microorganisms. This study relies on using low quality date syrup as a fermentation medium to produce bacterial cellulose. Previous studies have reported production of bacterial cellulose from other kinds of carbon sources to produce this product. Hungund *et al*. (2010 studied the production of bacterial cellulose by *Enterobacter amnigenus* using different nitrogen sources. They reported that using 0.5% casein hydrolysate could increase the yield of BC production to 2.8 g/l. This amount of production was obviously less than the BC produced by *G. xylinus* in both sucrose and date syrup fermentation media (1.69 and 4.35 g/100 ml of fermentation medium, respectively). Molasses could increase BC production (1.75 g/100 ml fermentation medium) by *G. xylinus* ATCC 10245 when added at concentration of 2% (Keshk *et al*., 2006). This production level was approximately 2.5 times less than the yield of BC produced by the date syrup fermentation carried out in this investigation. The T-test results showed that the yield of the BC produced in the date syrup fermentation medium was significantly different ($p<0.001$) relative to that for the sucrose medium. The X-ray patterns obtained using X-ray diffractometry represented the same chemical structure for both the BC and standard cellulose. The BC from produced date syrup showed less crystallinity (60.73%) when compared to standard cellulose (83.61%). Also, according to the FT-IR spectra intermolecular hydrogen bonds in BC were weaker than those in the standard cellulose. Low cristallinity and intermolecular hydrogen bonds in the BC compared to standard cellulose could make it chemically a more reactive component when participating in a chemical reaction (Fennema, 1997). The higher delicacy of the BC fibers as compared to cellulose (according to SEM images) makes it a suitable component for various applications in food systems. It is especially used when low-level use, lack of flavor interactions, foam stabilization, and stability over a wide range of pH, temperature, and freeze-thaw conditions are required (Khan *et al*. 2007). In conclusion, this study obviously showed the high capacity of low quality date syrup to be used as a fermentation medium for the production of BC by *G. xylinus*.

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