

Full Length Research Paper

Effect of various carbon and nitrogen sources on cellulose synthesis by *Acetobacter lovaniensis* HBB5

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The effect of various carbon and nitrogen sources on cellulose production by *Acetobacter lovaniensis* HBB5 was examined. In this study, glucose, fructose, sucrose and ethanol as carbon source and yeast extract, casein hydrolysate and ammonium sulphate as nitrogen source were used. Among the carbon sources, glucose gave the highest yield, followed by fructose, sucrose and ethanol. Besides, among the nitrogen sources, yeast extract gave the highest yield, followed by casein hydrolysate and ammonium sulphate. In Hestrin-Schramm (HS) medium that contained 2% (w/v) glucose and 0.5% yeast extract, bacterial cellulose production was 0.040 g/l (dry weight) after 7 days. Morphological view of *A. lovaniensis* HBB5 strain and the bundle structure of cellulose which were produced at optimum conditions were monitored by scanning electron microscopy. From TLC analysis, glucose was found as the main content of the bacterial cellulose monosaccharide. Moreover, the chemical structure of the bacterial cellulose was examined by FT-IR and NMR spectrophotometers. FT-IR and NMR spectrophotometry revealed that, all the bacterial cellulose samples were highly crystalline and were of cellulose type I.

Key words: *Acetobacter*, cellulose, production, characterization.

INTRODUCTION

Cellulose is the most abundant biopolymer on earth. Cellulose forms the cell wall of eukaryotic plants, algae and fungi and is synthesized by a variety of bacteria, including *Acetobacter xylinum* (Skinner and Cannon, 2000). *A. xylinum* is a rod shaped, aerobic, gram negative bacterium occurring as a contaminant in vinegar fermentation (Ramana et al., 2000).

Bacterial cellulose is an extracellular insoluble polysaccharide produced by some strains of *Acetobacter*. Cellulose synthesis occurs as a multi-step series of chemical reactions beginning with glucose that is catalyzed by enzymes (Jonas and Farah, 1998). Recently, different carbon sources, such as monosaccharides, oligosaccharides, alcohol and organic acids, were used to develop the bacterial cellulose (BC) production (Masaoko et al., 1993; Ishihara et al., 2002;

Son et al., 2003; Keshk and Sameshima, 2005; Mikkelsen et al., 2009; Jung et al., 2010). The glucose subunits that form the cellulose microfibril are extruded through pores in the cell wall of the bacteria. The pellicle floats on the surface of the medium allowing bacteria to obtain plenty of oxygen, which they require for growth, multiplication and more cellulose synthesis (Skinner and Cannon, 2000).

Investigations have been focused on the mechanism of biopolymer synthesis, as well as on its structure and properties, which determine practical use (Ross et al., 1991). Bacterial cellulose produced by *A. xylinum* is different from plant cellulose with respect to its unique physical and chemical properties (Watanabe et al., 1998). Plant cellulose contains both hemicellulose and lignin but bacterial cellulose consists of pure cellulose with its diameter of a thousandth of that of plant cellulose. One of the most important features of bacterial cellulose is its chemical purity, which distinguishes this cellulose from that from plants, usually associated with hemicelluloses and lignin, removal of which is inherently difficult (Delmer,

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1999).

Because of the unique properties resulting from the ultrafine reticulated structure, bacterial cellulose has found a multitude of applications in paper, textile and food industries, and as a biomaterial in cosmetics and medicine, artificial skin, artificial blood vessels, high-fidelity speakers and multifunctional sheets (Ring et al., 1986).

In this research, potential use of glucose, fructose, sucrose, ethanol as carbon source and also yeast extract, casein hydrolysate, ammonium sulphate as nitrogen source for bacterial cellulose production using *Acetobacter lovaniensis* HBB5 was tested. Some microstructural, hydrolysis products and chemical structure of the bacterial cellulose (BC) produced from glucose and yeast extract as carbon or nitrogen source were also investigated. This is the first report on bacterial cellulose (BC) production from *A. lovaniensis* HBB5 under static culture.

MATERIALS AND METHODS

Microorganism

A. lovaniensis HBB5 strain used in study was isolated from vinegar in Turkey (Swings, 1992; Holt et al., 1994). It was identified by using 16S rDNA complete sequencing method (Sacchi et al., 2002).

Culture media

Hestrin-Schramm medium (HS) was used as the basic medium. The HS medium for growth and cellulose production was composed of 20 g/l glucose, 5 g/l yeast extract, 5 g/l polypeptone, 2.7 g/l Na₂HPO₄ and 1.15 g/l citric acid (Hestrin and Schramm, 1954). The pH of the HS medium was adjusted to 6.0 using 1.0 M HCl.

Cultivation

For the preculture, stock culture was inoculated into 50 ml of the basal medium in a 250 ml erlenmeyer flask and culture was allowed to proceed at 30°C for 48 h under static conditions. The culture was started by inoculating 5% of the culture supernatant of a preculture. For static-flask fermentation, cultivations were performed at 30°C for 7 days under static conditions (Son et al., 2001).

Effect of carbon and nitrogen sources on cellulose production

Different carbon and nitrogen sources were used to test their effect on cellulose production. The carbon sources selected for their suitability were glucose, sucrose, fructose and ethanol at a concentration of 20 g/l along with 5.0 g/l yeast extract, 5.0 g/l polypepton, 0.675 g/l Na₂HPO₄ and 0.115 g/l citric acid. Furthermore, the following nitrogen sources were used by choosing each of the above carbon sources: casein hydrolysate and ammonium sulphate at a concentration of 5.0 g/l, instead of the yeast extract of the HS medium (Ramana et al., 2000).

Purification

After cultivation, the culture medium was separated into the

supernatant and the cellulose floccules by centrifugation at 3000 x g (Heraeus Sepatech Labofuge 200, Germany) for 15 min at room temperature. The cellulose floccules were washed with distilled water to remove medium components and treated with 4% (w/v) sodium hydroxide solution at 80°C for 1 h to eliminate bacterial cells. The bacterial cellulose was rinsed extensively with 6% acetic acid and then with distilled water until the pH of water became neutral (Ishihara et al., 2002). The purified bacterial cellulose was dried to constant weight at 105°C and then, weighed.

Scanning electron microscopy

Scanning electron microscopy of cellulose and cells were prepared according to Chávez-Pacheco et al. (2005). The samples were viewed on scanning electron microscope with JEOL /JSM-6335F from Tubitak, Turkey.

Analytical methods

The bacterial cellulose concentration in the culture broth was calculated from the dry weight of the purified cellulose. This was determined by the phenol/sulfuric acid method for total sugar using glucose as standard and absorption measurements at 470 nm using spectrophotometer (Shimadzu UV-1700, Japan) (Dubois et al., 1956). Output coefficient of specific product was calculated by the formula, according to Gerhardt and Drew (1994).

Thin-layer chromatography (TLC) of hydrolysis products

The purified polysaccharides (2 mg) were hydrolyzed in an aqueous solution of 2 M trifluoroacetic acid (TFA) for 2 h at 120°C in glass vial (Deeraksa et al., 2005). TLC of the hydrolysis products was made according to Fontana et al. (1988).

FT-IR spectroscopy

The thin samples of bacterial cellulose were prepared according to Kai and Keshk (1999). FT-IR spectra were recorded on Varian 800 infrared spectrometer (Shimadzu Corp., Japan).

CP/MAS ¹³C NMR analysis

CP/MAS ¹³C NMR measurements were conducted on the freeze-dried sample using a Bruker Biospin Ultrashield TM (300 MHz) spectrometer by the method of Yamamoto et al. (1996).

RESULTS AND DISCUSSION

Identification of microorganism

The neighbor-joining tree of partial (341 bp) 16S rDNA sequence of *Acetobacter* sp. HBB-5 obtained in this study together with selected sequences downloaded from GenBank is shown in Figure 1. The sequence analysis for the 16S rDNA gene of the isolate *A. lovaniensis* HBB5 was submitted to BLAST at the GenBank to find similar nucleic acid sequences. The 16S rRNA gene sequence of *A. lovaniensis* HBB5 was deposited in the GenBank under the accession number HQ108108.

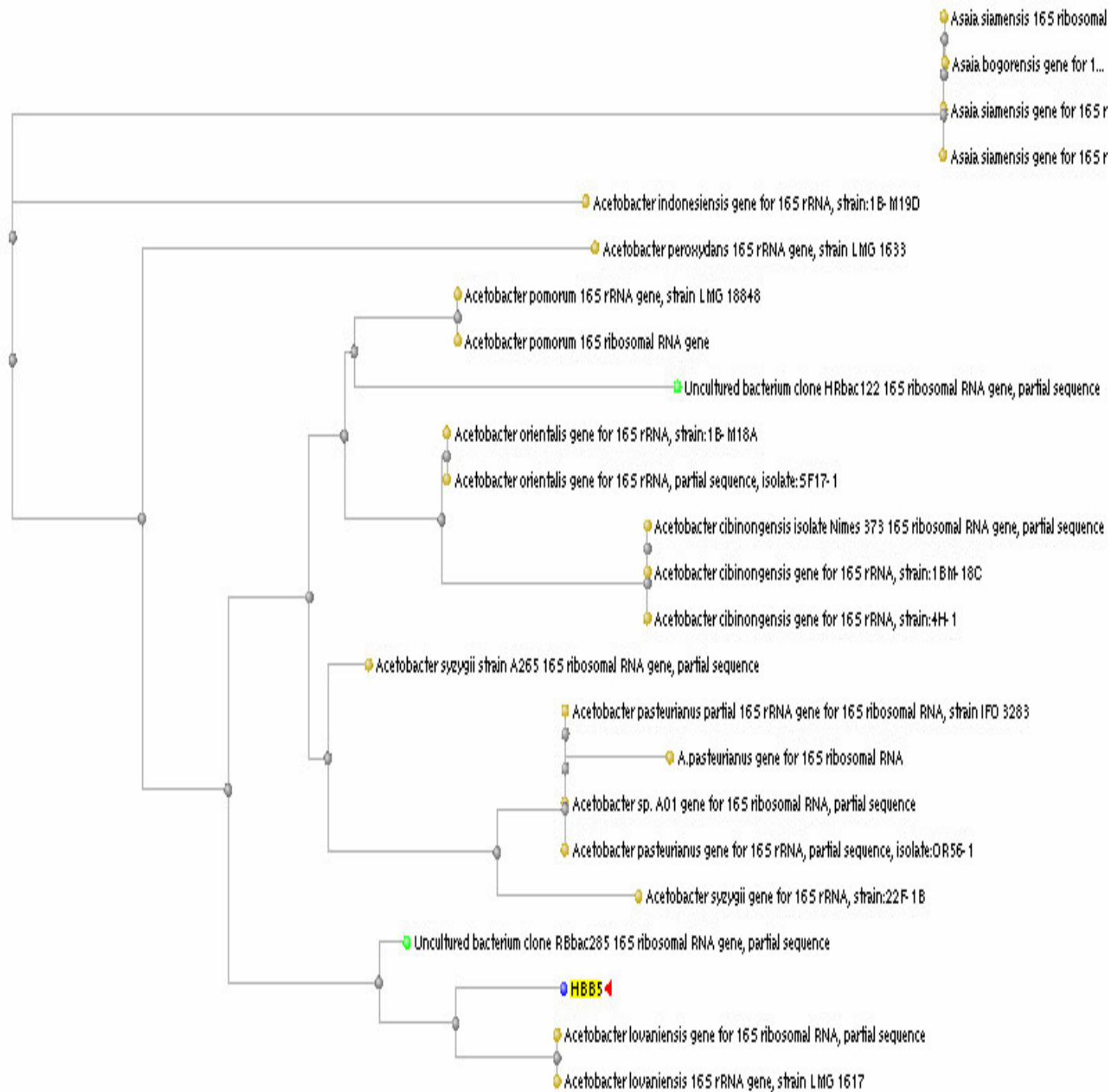


Figure 1. Neighbour-joining tree showing the phylogenetic affiliation of *Acetobacter* HBBS.

Effect of carbon and nitrogen sources on cellulose production

The cellulose producing output by *A. lovaniensis* HBB5 strain for the different carbon and nitrogen sources used, are shown in Table 1. From Table 1, the cellulose producing output changed from medium to medium; in the

medium that contained glucose or ammonium sulphate, cellulose production by *A. lovaniensis* HBB5 was about 3.6-fold lowest than that of glucose or yeast extract medium.

In the medium that contained glucose and casein hydrolysate, cellulose production was about 2.6-fold higher than that of the medium that contained glucose

Table 1. Effect of different carbon and nitrogen source on cellulose production by *A. lovaniensis* HBB5. Cells were cultivated in a flask-static at 30°C for 7 days.

Medium composition	Dry weight of cell (g/l)	Polysaccharide quantify according to standard curve at 470 nm (g/l)	Dry weight of raw cellulose (g/l)	Y(p/x)	Cellulose producing output (%)
Glucose + yeast extract (HS medium)	0.31	1810	0.040	0.129	12.9
Glucose + casein hydrolysate	0.24	1262	0.029	0.121	12.1
Glucose + ammonium sulphate	0.12	7120	0.011	0.092	9.2
Sucrose + yeast extract	0.29	1260	0.029	0.100	10.0
Sucrose + casein hydrolysate	0.32	1215	0.023	0.072	7.2
Sucrose + ammonium sulphate	0.37	1245	0.026	0.070	7.0
Fructose + yeast extract	0.29	1441	0.035	0.121	12.1
Fructose + casein hydrolysate	0.28	1408	0.031	0.111	11.1
Fructose + ammonium sulphate	0.30	1240	0.026	0.087	8.7
Ethanol + yeast extract	0.31	1237	0.025	0.081	8.1
Ethanol + casein hydrolysate	0.30	1228	0.021	0.070	7.0
Ethanol + ammonium sulphate	0.38	1206	0.019	0.050	5.0

Y(p/x), Output coefficient of specific product; p, dry weight of bacterial cellulose (g/l); x, dry weight of cell (g/l).

and ammonium sulphate. Furthermore, in the medium that contained glucose and yeast extract, cellulose production was about 1.6 times higher than that of ethanol and yeast extract. In the medium that contained fructose and yeast extract, cellulose production was about 1.1-fold lower than that of glucose and yeast extract. In addition, in the medium that contained fructose and yeast extract, cellulose production was about 1.4-fold higher than that of ethanol and yeast extract. In the medium with sucrose and yeast extract, cellulose production was about 1.4-fold lower than that of glucose and yeast extract. Furthermore, in the medium with sucrose and yeast extract, cellulose production was about 1.6-fold higher than that of ethanol and yeast extract. In the medium that contained fructose and casein hydrolysate, cellulose production was about 1.2-fold higher than that of fructose and ammonium sulphate and was about 1.1-fold lower than that of fructose or yeast extract. In the medium that contained sucrose and casein hydrolysate, cellulose production was about 1.1-fold lowest than that of sucrose and ammonium sulphate and was about 1.3-fold lower than that of sucrose and yeast

extract. In the medium that contained ethanol and casein hydrolysate, cellulose production was about 1.1-fold higher than that of ethanol and ammonium sulphate and was about 1.2-fold lower than that of ethanol and yeast extract.

In recent years, various carbon sources including monosaccharides, oligosaccharides, alcohols, sugar alcohols and organic acids, were used to maximize the bacterial cellulose production by various *Acetobacter* strains (Yang et al., 1998; Ishihara et al., 2002; Keshk and Sameshima, 2005; Jung et al. 2010). Glucose, sucrose and mannitol were found to be the optimal carbon sources for cellulose production by *A. xylinum* NCIM 2526 (Ramana et al., 2000). *G. hansenii* PJK (KCTC 10505 BP) produced 1.72 g/l of cellulose when glucose was provided as the carbon source (Park et al., 2003). *Acetobacter* sp. V6 strain isolated from the traditionally fermented vinegar produced 4.16 g/l cellulose in a complex medium containing glucose as a carbon source (Son et al., 2003). Jung et al. (2010) used glycerol as a carbon source for the production of cellulose by *Acetobacter* sp. V6 and obtained a yield of 4.98 g/l after 7

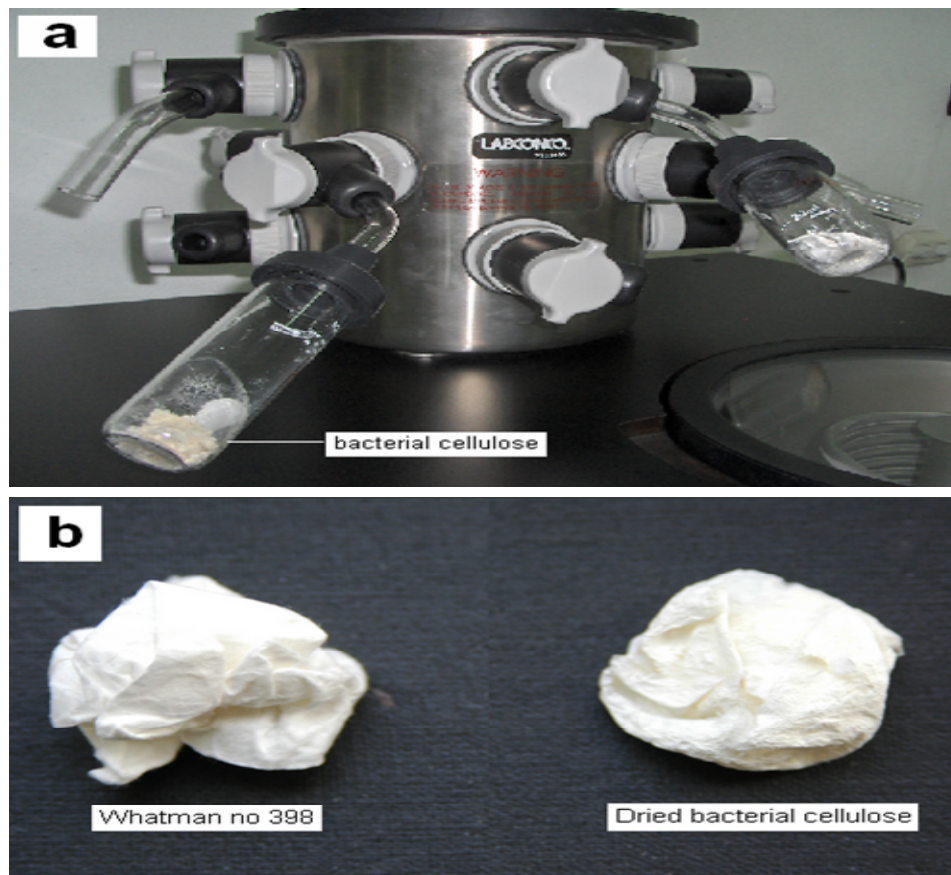


Figure 2. Lyophilization of bacterial cellulose. The dried bacterial cellulose (a) was compared with Whatman no 398 (b).

days.

Masaoka et al. (1993) reported that the yield of cellulose, relative to the amount of glucose consumed, decreased with increase in the initial glucose concentration. Bacterial cellulose production was enhanced with increasing amount of glucose of up to 1.5%, but decreased when it was about 2% of glucose (Son et al., 2003). However, Keshk and Sameshima (2005) reported that the maximum yield was obtained at 1% concentration, whereas, the minimum was observed at both 2 and 3% concentrations.

Bacterial cellulose production was strongly affected by the ethanol concentration of the medium. When the added ethanol was more than 1% (v/v), cellulose production decreased (Park et al., 2003). Naritomi et al. (1998) reported that, an increase in ethanol concentration to more than 1.5% (v/v) decreased the cellulose production rate due to the inhibition of the growth of *A. xylinum* subsp. *sucrofermentans* BPR 2001 by acetate that was generated.

The effect of various nitrogen sources on the production of bacterial cellulose has been reported; casein hydrolyzate gave yield of 5 g/l and peptone gave yield of 4.8 g/l of cellulose in *A. xylinum* (Ramana et al., 2000).

Son et al. (2003) observed various nitrogen sources which were added separately to the medium at 0.5% (w/v) to assess their effects on cellulose production. Among them, yeast extract was the best nitrogen source for cellulose production by *Acetobacter* sp. A9. Matsuoka et al. (1996) observed that, lactate had a stimulating effect on cellulose production when it was added with 4% (by mass per volume) fructose containing corn steep (liquor, yeast extract or peptone as a nitrogen source).

Morphology of bacterial cellulose

As seen in Figure 2a, the purified cellulose floccules were lyophilized (Labconco, USA). The dried bacterial cellulose compared with Whatman No 398 was shown in Figure 2b. Besides, the SEM images of BC produced from the glucose media is shown in Figure 3a, b.

It was reported that, morphological changes of the bacterial cellulose were related to the differences in microstructure, namely, crystallinity and average degree of polymerization (Watanabe et al., 1998). The fibrils of BC from the glycerol medium were entangled with each other resulting in a denser reticulated structure than

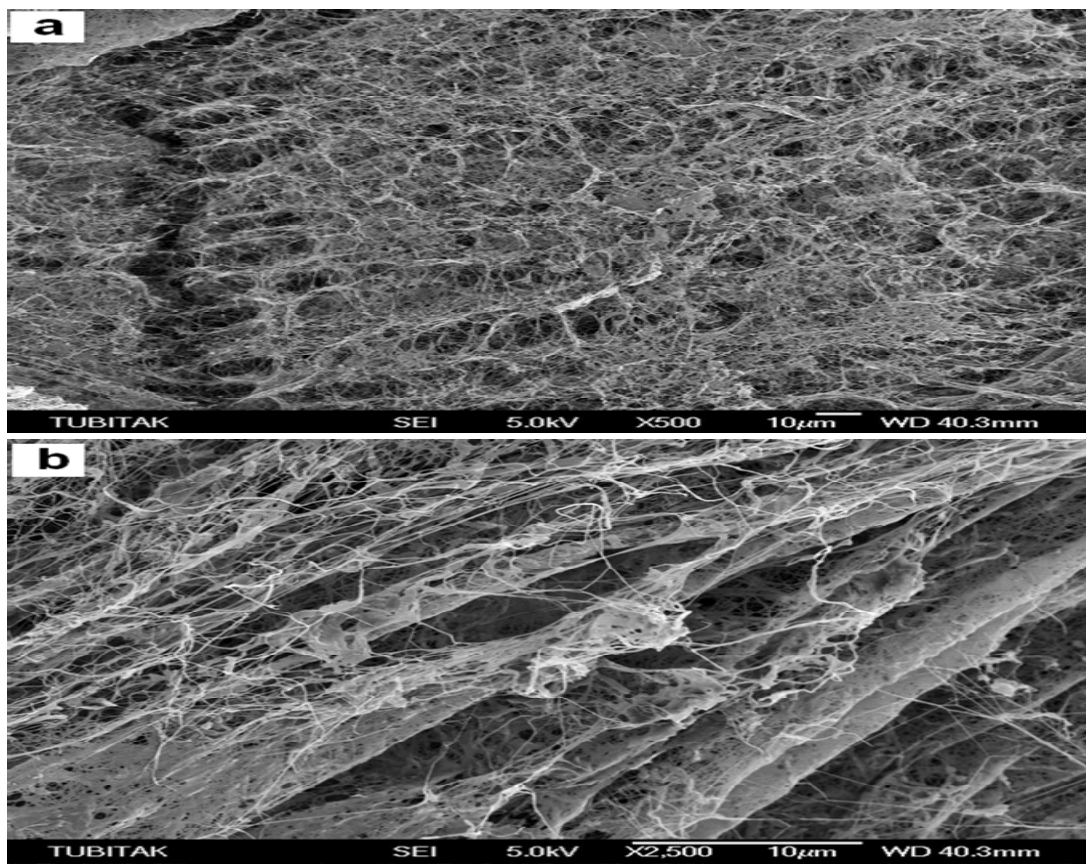


Figure 3. Scanning electron micrographs of the surface structure of the bacterial cellulose (a, magnification x 500 and b, magnification x 2.500).

those from the glucose medium (Jung et al., 2010).

Thin-layer chromatography (TLC) of hydrolysis products

In order to examine the composition of the purified bacterial cellulose, TLC was carried out after acid-hydrolysis with 2 N TFA, as described in the materials and methods. As shown in Figure 4, there was one detectable sugar in the acid-hydrolysis product of the purified cellulose of the *A. lovaniensis* HBB5 strain. The sugar detected in the acid-hydrolysis, corresponded to glucose.

FT-IR spectroscopy

As shown in Figure 5, the conformational characteristics of the bacterial cellulose from the glucose media were determined by FT-IR spectroscopy. The FTIR spectra showed characteristic cellulose peaks around 3430 to 3435 cm^{-1} for hydroxyl groups stretching vibration, at 2927 to 2949 cm^{-1} for C-H stretching vibration, at 1433 to 1456 cm^{-1} for C-H bending vibration and at 1045 to 1067

cm^{-1} for C-O-C and C-O-H stretching vibration of the sugar ring (Socrates, 2001; Sun et al., 2007).

In the FT-IR spectra of cellulose, the band at 3400 cm^{-1} was attributed to the intramolecular hydrogen bond for 3O...H-O5 (Oh et al., 2005, Jung et al., 2010). It is particularly useful for elucidating hydrogen-bonding patterns because, in favorable cases, each distinct hydroxyl group gives a single stretching band at a frequency that decreases with increasing strength of hydrogen bonding (Sturcova et al., 2004).

CP/MAS ^{13}C NMR analysis

The CP/MAS ^{13}C NMR spectra of the bacterial cellulose is shown in Figure 6. In the spectrum of the bacterial cellulose, the enhanced downfield resonance line for the C4 triplet at 85 and 80 ppm and the strong central resonance line for the C1 triplet at 100 ppm indicates that, cellulose I $_{\alpha}$ was dominant in this sample (Watanabe et al., 1998).

Atalla and Van der Hart (1984) used solid state NMR to determine the native cellulose structure called cellulose I and was composed of two crystal forms, cellulose I $_{\alpha}$ and cellulose I $_{\beta}$.

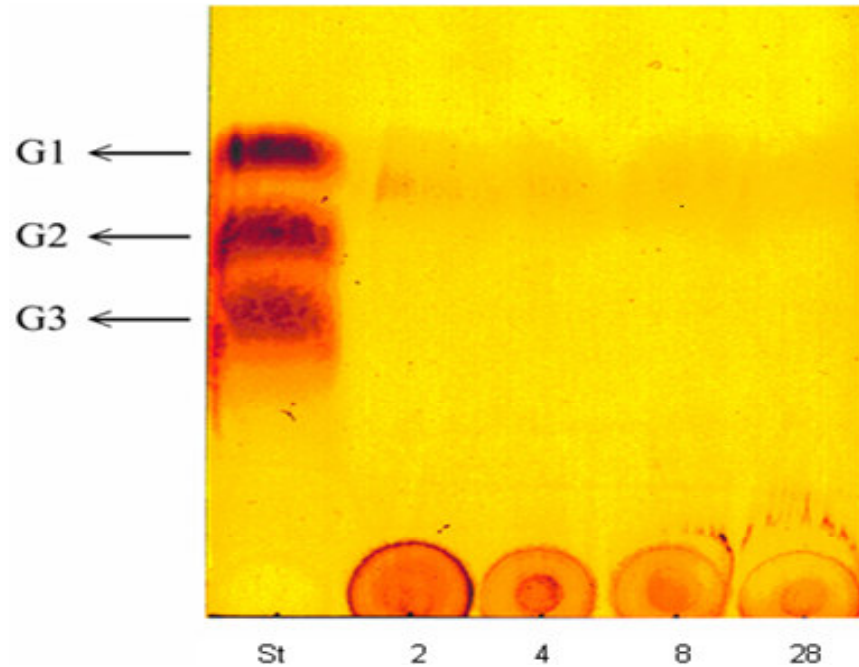


Figure 4. Thin-layer chromatography of the reaction product of purification of cellulose from *A. lovaniensis* HBB5 strain. Hydrolysis time were 2, 4, 8 and 28 h. A mixture of glucose and celooligosaccharides was used as standards (St): glucose (G1), cellobiose (G2) and cellotriose (G3).

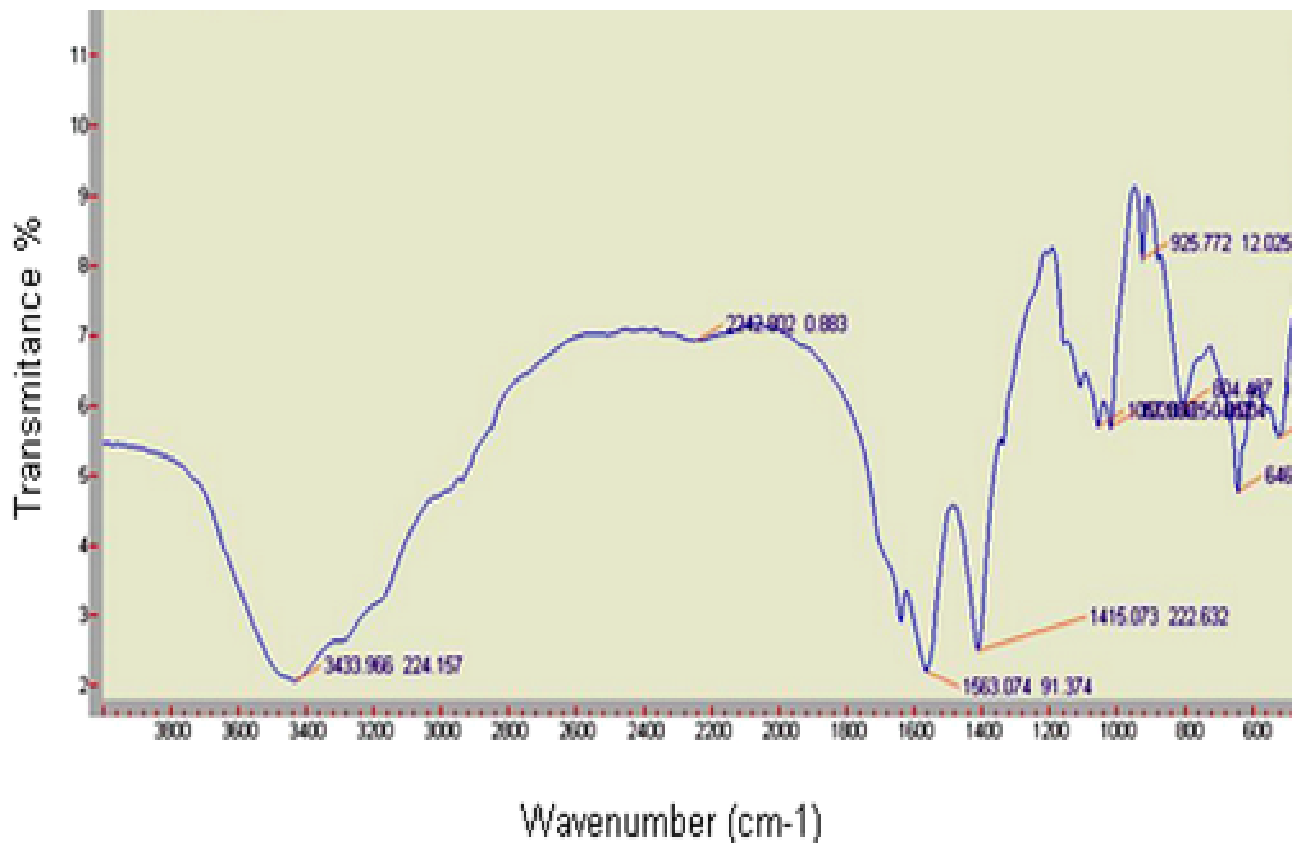


Figure 5. FT-IR spectroscopy of bacterial cellulose.

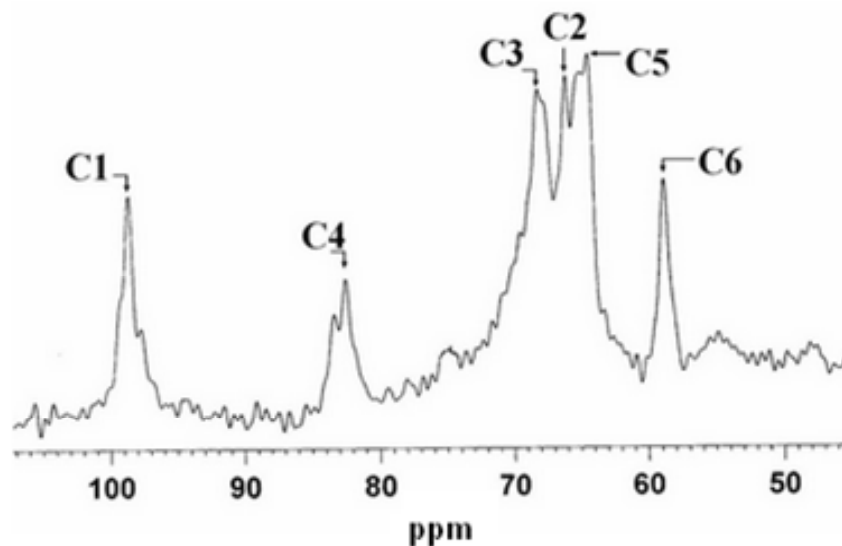


Figure 6. CP/MAS ^{13}C NMR spectra of bacterial cellulose.

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