Analytical Investigations of Bacterial Cellulose

*Ulrike Udhardt, Stephanie Hesse, Dieter Klemm**

Institute of Organic Chemistry and Macromolecular Chemistry
Friedrich Schiller University of Jena,
Humboldtstrasse 10, D-07743 Jena, Germany

Summary: The cultivation of the bacterium *Acetobacter xylinus* AX 5 was carried out in the common Hestrin-Schramm medium containing D-glucose as C-source and citric acid as buffer component. HPLC studies proved to be convenient methods to investigate the stability and interactions of these constituents in the starting culture liquid. Within the initial sterilization step and limited by the citric acid, up to 6 % of the D-glucose was partially isomerized to D-fructose and degraded to dark-yellow products. In static culture, *A. xylinus* AX 5 produces cellulose extracellularly on the surface of this medium. Solid-state NMR spectroscopy represents a suitable analytical method to characterize the supramolecular structure of the bacterial cellulose in never-dried, air-dried, and freeze-dried states. It could be demonstrated that the drying process reduces the degree of crystallinity in the range of about 12 % without changes in the Iα/β ratio of these cellulose modifications.

Keywords: *Acetobacter xylinus*; analytical investigations; bacterial cellulose; high performance liquid chromatography (HPLC); NMR

Introduction

Acetobacter xylinus (*A. xylinus*), a rod shaped, aerobic, gram negative bacterium, produces a white gelatinous material (pellicle) on the surface of the culture liquid in static culture. In 1886, Brown[1] first reported that the pellicle was composed of pure cellulose called "bacterial cellulose". During the last 10 years, in the field of cellulose research and development growing activities on bacterial cellulose can be observed.

The cellulose synthesized by *A. xylinus* is quite different from those of plant celluloses. That especially concerns the ultrafine network architecture, high crystallinity and hydrophilicity, purity (free of lignin and other biogenic byproducts) and mouldability during formation[2-5]. Because of the special synthetic strategy of the microorganisms, analytical investigations of bacterial cellulose include both studies of the culture medium and studies of the formed products.

Kinetic investigations of substrate utilization, cellulose formation, and metabolic processes in different culture media are discussed detailed in literature using enzymatic[6,7] or

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chromatographic (HPLC) methods[2,4,8-11]. In connection with these investigations, analytical studies of the starting culture liquid (not inoculated culture medium) play a rather minor role. However, the knowledge about stability and interactions of the components of the culture liquid, especially during the essential sterilization step, should be the basis for interpretation of kinetic studies mentioned above.

Analytical investigations of the cellulose product synthesized in the culture medium are directed to the morphological, molecular, and supramolecular structure. Whereas the morphological structure could be disclosed by scanning electron microscopy, the molecular structure is, e.g., characterized by degree of polymerization, polydispersity, and content of carbonyl groups[12]. As a powerful tool to investigate the supramolecular structure of cellulose, solid-state CP/MAS ^{13}C-NMR has been shown[13]. Resulting from these investigations, naturally occurring cellulose I can be subdivided into cellulose Iα most abundant in lower plants and bacteria and cellulose Iß mostly found in higher plants[14-16]. As cellulose Iα is a triclinic phase consisting of one chain per unit cell[17,18] and a cellobiose repeat with two different anhydroglucose rings per crystallographically unique chain[19], twelve resonances should be observed by ^{13}C-NMR in case of the Iα-modification. The same applies for polymorph Iβ. Similarly, Sugiyama et al.[17,18] found that cellulose Iβ is monoclinic with two crystallographically inequivalent chains per unit cell, whereas the units in each of the chains are equal but different for the two chains[20]. The first assignment of the ^{13}C chemical shifts (CS) of C1 (96 to 108 ppm), C4 (81 to 93 ppm), and C6 (60 to 70 ppm) has been published by VanderHart and Atalla[21]. The overlapping resonances between 70 and 80 ppm were attributed to the carbon atoms C2, C3, and C5 without further identification. This assignment was confirmed later using selectively ^{13}C labeled cellulose[22,23] and using solid-state INADEQUATE NMR[24]. In both cases the C2, C3, and C5 chemical shifts have been determined as well. For both modifications, Kono et al.[22] found experimentally that only two carbon signals appear for each carbon site in the structure. Exceptions are C1 and C6 of cellulose Iα and C2 of cellulose Iβ with identical chemical shifts for the respective sites. The same authors assigned all carbon signals to the carbon sites in the two different anhydroglucose rings of purified *Cladophora* (cellulose Iα) and *tunicate* (cellulose Iβ)[25]. Similarly, for bacterial cellulose the line assignment has been carried out using uniformly ^{13}C enriched material (AX-DSM 13368)[26] and furthermore, the assignments of the ^{13}C resonances of cellulose

Iα were reinvestigated performing refocused INADEQUATE NMR on uniformly ^{13}C enriched celluloses of different strains (ATCC 53582, ATCC 23769)[27-29]. Basically, it turned out that differences in the isotropic chemical shifts of the various carbons exist for the various origins of the cellulose material. The supramolecular structure of never-dried bacterial cellulose (NCIB 8034, NCIB 8246) has been investigated by Fink et al.[30] achieving a model of never-dried swollen microfibrillar ribbons of 5 to 12 water free Iα-crystalline subunits and of water solvating the subunits. These crystalline units are aggregated laterally along the (110)-lattice planes with a layer of water between adjacent crystallites, whereas NMR investigations indicated cellulose-water interactions. During air-drying, the Iα/β ratio of about 80/20 remained approximately unchanged, while the overall crystalline order decreased. These results have inspired structure analyses of bacterial cellulose of the strain AX-DSM 14666 in dependence on the water content of the sample and on different drying methods.

In a first section, the present paper deals with the analysis of the starting Hestrin-Schramm medium (HS-medium) using enzymatic (GOD-POD: glucose oxidase-peroxidase system) and chromatographic (HPLC) methods. The studies are directed to the stability of the C-source D-glucose and the role of citric acid during the sterilization process.

The second part describes the supramolecular structure of bacterial cellulose isolated from the HS-medium by solid-state NMR spectroscopy. In detail, the special feature of never-dried products in comparison to the air-dried and freeze-dried variant is discussed.

Experimental

Cultivation conditions: The bacterium *Acetobacter xylinus* AX 5 (strain collection of the Institute of Organic Chemistry and Macromolecular Chemistry Jena, AX-DSM 14666; DSM: Deutsche Sammlung für Mikroorganismen und Zellkulturen Braunschweig) was cultivated in Erlenmeyer flasks containing Hestrin-Schramm medium[31] in static culture at 28 °C for 10-14 d. 20 ml of the medium was inoculated with 1 ml of a 14 d old liquid pre-culture.

Sterilization: Steam sterilization of the culture liquid was carried out in a pressure cooker at 117 °C for 20 min.

Isolation and purification: The cellulose pellicles were taken from the culture medium, washed with distilled water, treated with boiling 0.1 N aqueous sodium hydroxide for

30 min, and washed with distilled water to a neutral reaction of the rinsing agent.

Enzymatic determination of D-Glucose: The glucose oxidase-peroxidase (GOD-POD) test system Fermognost®, GERMED has been used.

HPLC: A KNAUER HPLC equipment consisting of a pump, an injection valve with a 20 µl sample loop, a differential refractometer and UV-detector was used. The measurements were carried out with a Biorad® system (BIORAD AMINEX 87H, 300x7, 8 mm, 9 µm) at 65 °C with 0.01 N H_2SO_4 as the mobile phase and a flow rate of 0.5 ml/min. Samples of about 1 ml of the culture medium were centrifuged at 6.000 rpm for 5 min and diluted with water.

NMR spectroscopy: CP/MAS ^{13}C-NMR and MAS ^{1}H-NMR spectra were recorded using a Bruker AMX 400 spectrometer operating at 100.58 MHz and 400.13 MHz, respectively, with a 4 mm MAS double resonance probe and ZrO_2 rotors. The sample spinning frequency was 6.5 kHz and 12 kHz, respectively. In ^{13}C-NMR, the repetition time was 2 s and the cross polarization time was 1 ms. Furthermore, two-pulse phase modulation (TPPM: ± 10°, 7 µs) has been applied for proton decoupling and adamantane was used as an external reference having ^{13}C chemical shifts of 29.50 ± 0.10 ppm (CH) and 38.56 ± 0.10 ppm (CH_2) with respect to tetramethylsilane at 0.0 ppm[32]. MAS ^{1}H-NMR measurements have been carried out at different repetition times (0.5, 1, 1.5, 2, etc. ... 15 s) to determine the proton spin-lattice relaxation times of the various cellulose samples by line shape analysis.

Results and Discussion

Investigation of the Stability of D-glucose in the HS-medium under Sterilization Conditions by HPLC

The liquid Hestrin-Schramm medium (HS-medium) is the common culture medium for cultivation of *A. xylinus* in static culture. In the unsterilized form, it contains D-glucose as C-source, peptone as N-source, yeast extract as vitamin source, citric acid and sodium hydrogen phosphate as the so called McIlvain buffer system[31,33] with the detected pH-value of 6.1 (Table 2).

The enzymatic determination of the concentration of D-glucose in the culture liquid before and after steam sterilization demonstrates a loss of D-glucose in the range of about 6 %

confirmed by HPLC investigations, too. Moreover, the culture medium shows a dark-yellow color. Table 1 shows the concentration of D-glucose before and after sterilization. Table 2 contains information on the change of pH-value and color. It is well known that D-glucose isomerizes to D-fructose and D-mannose in an aqueous alkaline environment based on a tautomeric equilibrium known as Lobry-de-van-Ekenstein-rearrangement[34,35]. A transformation of D-glucose to D-fructose already occurs by boiling the aqueous solution with alkaline earth carbonates[36]. With the help of HPLC, the coexistence of D-glucose and D-fructose in the sterilized HS-medium could be detected (Figure 1).

Moreover, by heating of D-glucose in presence of disodium phosphate, methylglyoxal and acetic acid are formed[36]. The 1,2-dicarbonyl compound (not detected by HPLC) seems to be the reason for the colouring of the HS-medium after sterilization. Traces of acetic acid (not detected by HPLC) could explain the decrease of the pH-value down to 5.7 after sterilization (Table 2).

Table 1. Determination of the concentration of D-glucose in the HS-medium.

Method	D-glucose	
	before sterilization	after sterilization
	mg/ml	mg/ml
GOD-POD test	22.88	21.50
HPLC	18.99	17.86

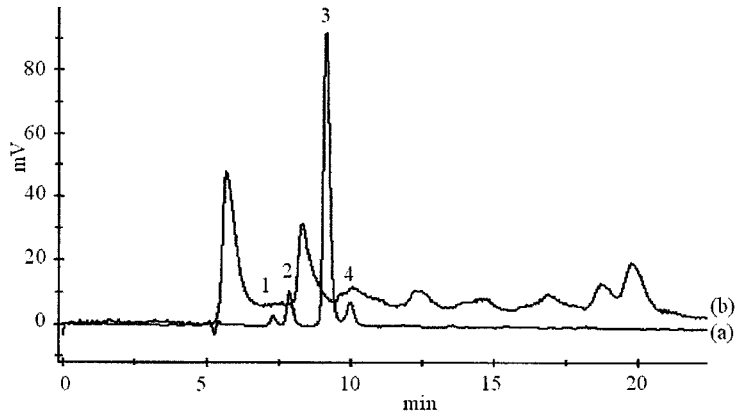

Figure 1. HPLC elugramm of the sterilized HS-medium (1: yeast extract; 2: citric acid; 3: D-glucose; 4: D-fructose; (a) RI detector, (b) UV detector).

As a result of systematic sterilization tests (Figure 2), the influence of the components of the culture medium on D-glucose loss and yellowing can be described. D-Glucose itself is relatively stable in aqueous solution without addition of the other components (Figure 2A). Whereas citric acid, peptone and yeast extract have no influence on the C-source, the addition of Na_2HPO_4 causes marked structural modifications (Figure 2B) and a drastic decrease of the pH-value (Table 2). By combination of D-glucose, Na_2HPO_4, and citric acid, the pH-value is adjusted in this way that changes in the composition of the medium are proportional low (Table 2, Figure 2C)[37].

Table 2. pH-value of the components of the HS-medium in distilled water.

Components	pH-value			
	before sterilization (color of the medium)		after sterilization (color of the medium)	
peptone	5.7	(yellow)	5.8	(yellow)
yeast extract (YE)	6.5	(light-yellow)	6.6	(light-yellow)
Na_2HPO_4	8.5	(colorless)	8.6	(colorless)
citric acid (CA)	2.9	(colorless)	2.9	(colorless)
D-glucose (Glc)	**6.8**	**(colorless)**	**6.7**	**(colorless)**
peptone + Glc	5.6	(yellow)	5.5	(yellow)
YE + Glc	6.4	(light-yellow)	6.1	(light-yellow)
CA + Glc	2.8	(colorless)	2.8	(colorless)
Na_2HPO_4 + Glc	**8.5**	**(colorless)**	**6.3**	**(dark-yellow)**
peptone + YE + Glc	6.1	(yellow)	5.9	(yellow)
peptone + Na_2HPO_4 + Glc	7.6	(yellow)	6.8	(orange)
peptone + CA + Glc	4.0	(yellow)	4.0	(yellow)
YE + Na_2HPO_4 + Glc	7.8	(light-yellow)	6.7	(orange)
YE + CA + Glc	4.1	(light-yellow)	4.1	(light-yellow)
Na_2HPO_4 + CA + Glc	**6.2**	**(colorless)**	**6.1**	**(light-yellow)**
peptone + YE + Na_2HPO_4 + Glc	7.4	(yellow)	6.7	(orange)
peptone + YE + CA + Glc	4.3	(yellow, cloudy)	4.3	(yellow, cloudy)
YE + Na_2HPO_4 + CA + Glc	6.3	(light-yellow)	6.1	(yellow)
peptone + Na_2HPO_4 + CA + Glc	6.1	(yellow)	5.9	(dark-yellow)
peptone+YE+CA+Na_2HPO_4+Glc (HS-medium)	**6.1**	**(yellow)**	**5.7**	**(dark-yellow)**

Time dependent HPLC studies of the HS-culture (sterilized and inoculated HS-medium) have shown that the small amount of D-fructose contained in the culture liquid was metabolized by *A. xylinus* AX 5 only after the complete consumption of D-glucose. Using

D-fructose instead of D-glucose as the sole C-source in the culture liquid, D-fructose is integrated in the cellulose biosynthesis after a longer adaptation period.

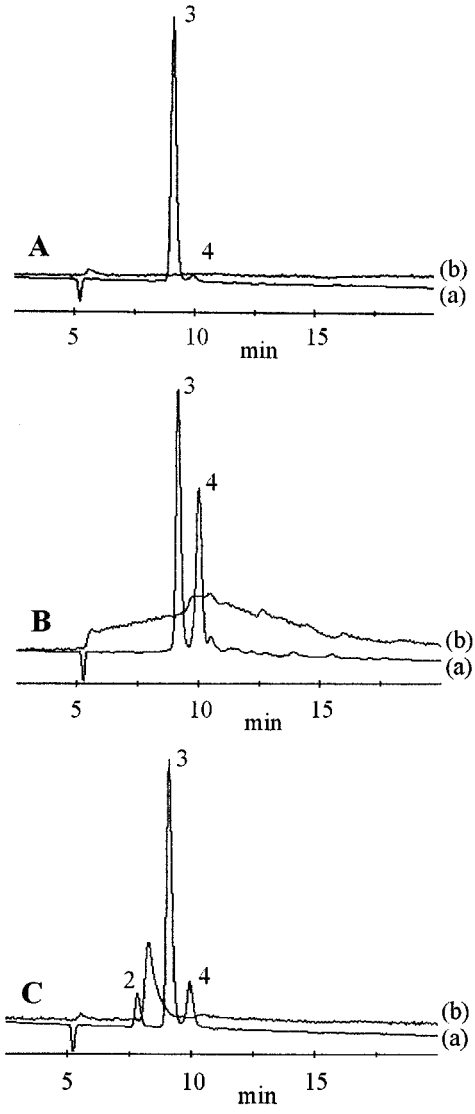

Figure 2. HPLC elugramms of sterilized aqueous solutions of (A) D-glucose, (B) D-glucose in presence of Na_2HPO_4, (C) D-glucose in combination with Na_2HPO_4 and citric acid (2: citric acid; 3: D-glucose; 4: D-fructose; (a) RI detector; (b) UV detector).

Premjet et al.[33] concluded from investigations of cellulose biosynthesis with different concentrations of the McIlvain buffer components and from studies of different buffer systems that the McIlvain buffer does not work effective as a pH-regulator. The authors discussed the function of citric acid as promoter of the cellulose biosynthesis. Jonas and Farah[3] determined a cellulose yield of 20 % in comparison with D-glucose starting from citrate as C-source. Contrary, Fiedler et al.[7] observed no growth on citrate.

In the course of our HPLC investigations of the inoculated HS-medium, we could further demonstrate that in contrast to earlier studies[38] using a co-culture of *A. xylinus* and *Enterococcus faecium,* citric acid does not serve as a C-source for *A. xylinus* AX 5 to produce bacterial cellulose. Using a citric acid-free HS-medium, we detected a marked change of pH-value and a drastic decrease of the concentration of D-glucose during the sterilization process (Table 3).

Table 3. Influence of citric acid on several cultivation parameters.

Cultivation parameters	HS-medium	
	with citric acid	without citric acid
pH-value		
before sterilization	6.3	7.4
after sterilization	6.0	6.5
D-glucose (HPLC)		
before sterilization	18.99 mg/ml	19.85 mg/ml
after sterilization	17.86 mg/ml	13.34 mg/ml

Investigation of the Supramolecular Structure of Bacterial Cellulose by Solid-State NMR Spectroscopy in Dependence on the Kind of Pellicle-Drying

Bacterial cellulose synthesized by *A. xylinus* AX 5 in form of pellicles at the air/liquid interface of the HS-medium is a high molecular compound (DP 4000-6000) with a remarkable uniform distribution of chain length and a construct of carbonyl groups according to the end groups. The microbial polysaccharide is characterized by ultrafine fibers (diameter < 1 μm) and network structure. During cultivation, the microorganisms produce cellulose in a high swollen form. The hydrophilicity of the cellulose pellicle is explained by presence of pore structures within the wet product. The water retention value (WRV) of the never-dried material is 1030 %. After air-drying of the bacterial cellulose

and re-swelling with water, the WRV was drastically decreased (110 %) and comparable with those of plant cellulose. Freeze-drying has been proven to be a more considerate drying method relating to the maintenance of pore structures (WRV: 630 %)[2,37].

Because of these features, there was an interest to investigate the samples mentioned above by solid-state NMR spectroscopy in order to answer the following question: Do structure parameters reflect the swelling properties?

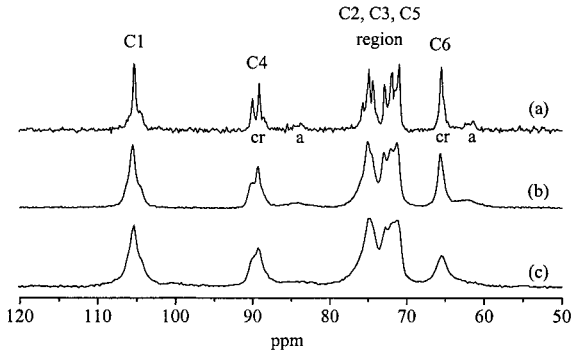

Figure 3. CP/MAS ^{13}C-NMR [100.58 MHz] spectra of (a) never-dried, (b) air-dried, and (c) freeze-dried bacterial celluloses (AX 5).

Figure 3 shows the CP/MAS ^{13}C-NMR spectra of a highly-ordered, never-dried bacterial cellulose (a), and of two medium-ordered, dried cellulose samples (b and c) clearly demonstrating the existence of crystalline cellulose I. As bacterial cellulose consists of a mixture of Iα and Iβ, the corresponding carbon resonances are found in the spectra, and besides, most of these lines are resolved in the ^{13}C-NMR spectrum of never-dried bacterial cellulose *A. xylinus* AX 5. For the never-dried sample the ^{13}C-chemical shifts could be assigned to the carbons of the crystalline parts as follows: C1(β) [106.2 ppm]; $C1_{1,2}(\alpha)$ [105.5 ppm]; C1'(β) [104.2 ppm]; $C4_1(\alpha)$ [90.2 ppm]; $C4_2(\alpha)$ & C4(β) [89.3 ppm]; C4'(β) [88.5 ppm]; C3'(β) [76.0 ppm]; $C3_1(\alpha)$ [75.0 ppm]; $C3_2(\alpha)$ & C3(β) [74.5 ppm]; $C5_2(\alpha)$ & C5(β) [72.9 ppm]; $C2_1(\alpha)$, C2(β), C2'(β) & C5'(β) [72.0 ppm]; $C2_2(\alpha)$ & $C5_1(\alpha)$ [71.1 ppm]; $C6_{1,2}(\alpha)$, C6(β) & C6'(β) [65.5 ppm]. At first view, the difference between the ^{13}C-NMR spectra of dried and never-dried bacterial celluloses is the larger line width for the carbon signals of the dried samples. Note, that in the wet state sharpness and resolution of the multi-component lines of the ^{13}C-NMR spectra are much

better than in the dried state, however the drying procedure does not shift the isotropic values.[39]

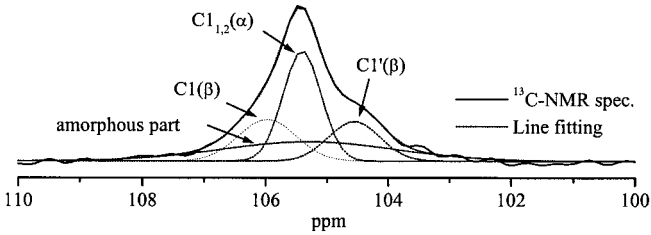

Figure 4. Line fit analysis of the enlarged C1 region of the CP/MAS ^{13}C-NMR spectrum of bacterial cellulose for quantifying the relative portions of α and β phases.

Using CP/MAS ^{13}C-NMR spectroscopy in combination with line fit analysis of the C1 resonances according to Figure 4, the cellulose I-type with a high content of the Iα-modification could be proved for the dried as well as never-dried samples (Table 4). Further, the quantitative analysis of the C4 resonances for the crystalline (around 90 ppm) and amorphous (at about 84 ppm) regions show, that only about ¼ of bacterial cellulose is found in amorphous parts. While the ratio of the Iα and Iβ modifications does not change significantly with the drying process, the degree of crystallinity x_c reduces in the range of about 12 % by freeze-drying of bacterial cellulose (Table 4). The C4 line fitting has been carried out according to the spectral fitting profiles of Tokoh et al.[40]

Table 4. Comparison of the ratio of the Iα and Iβ modifications, the degree of crystallinity x_c, and the proton spin-lattice relaxation times T_1^H of differently dried bacterial celluloses.

Bacterial cellulose	Ratio α : β	Amount of crystalline parts x_c	Proton spin-lattice relaxation time T_1^H
		%	s
freeze-dried	2.6 : 1	68	1.4
air-dried	2.7 : 1	72	2.0
never-dried	2.9 : 1	80	2.7

In addition, the proton spin-lattice relaxation times of the differently dried cellulose samples have been calculated by line-shape analysis of the ^{1}H-NMR spectra recorded at different repetition times. Relaxation measurements have utility for elucidating polymer dynamics in the solid-state. In MAS ^{1}H-NMR, efficient spin diffusion usually results in

averaging of relaxation behavior over the ensemble of protons to yield a single relaxation time for all protons. As we interpret the data regarding obvious differences in the differently dried samples, and not in terms of localized motion, this fact is not of significance for our investigations. Corresponding to the data in Table 4, the protons of never-dried bacterial cellulose are of reduced mobility compared to those of dried samples. This leads back to strong hydrogen bonds within the never-dried sample, and implies a hydrogen bonding system which is in better order[27]. According to Fink et al.[30], water solvated crystalline units aggregate laterally including water molecules between adjacent units, whereas lateral binding forces are constituted mostly by coordinated hydrogen bonds at the interface. Freeze-drying influences the crystallinity and the proton spin-lattice relaxation times of bacterial cellulose more than drying at air.

Conclusion

As part of investigations of cellulose formation by *A. xylinus* AX 5, the aim of the paper is to present first results on the stability of the most important C-source D-glucose in the HS-medium and on the influence of drying methods on the supramolecular structure of the synthesized bacterial cellulose. During the essential sterilization step of the culture liquid, D-glucose is partially isomerized to D-fructose and degraded to dark-yellow products. Citric acid as one component of the buffer system serves as a stabilizing agent for D-glucose during the sterilization process.

Investigating the synthesized cellulose pellicles, we could prove that structure parameters like degree of crystallinity and state of order determined by proton mobility are influenced by the water content of bacterial cellulose and the used drying method.

Therefore, the pre-treatment of the culture liquid and the after-treatment of the synthesized cellulose are sensitive parts of the biotechnological cellulose formation.

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