Journal of Industrial Microbiology & Biotechnology (2002) 29, 189–195

www.nature.com/jim

Factors affecting the yield and properties of bacterial cellulose

A Krystynowicz, W Czaja, A Wiktorowska-Jezierska, M Gonçalves-Miśkiewicz, M Turkiewicz and S Bielecki

Institute of Technical Biochemistry, Technical University of Lodz, Stefanowskiego 4/10, Lodz 90-924, Poland

***Acetobacter xylinum* E_{25} has been applied in our studies in order to find optimal culture conditions for effective bacterial cellulose (BC) production. The strain displays significantly higher stability in BC production under stationary culture conditions. In contrast, intensive agitation and aeration appear to drastically reduce cellulose synthesis since such conditions induced formation of spontaneous cellulose nonproducing mutants (*Cel–*), which dominated in the culture. Mutation frequency strictly depends on the medium composition in agitated cultures. Enrichment of the standard SH and Yamanaka media with 1% ethanol significantly enhanced BC production in stationary cultures. Horizontal fermentors equipped with rotating discs or rollers were successfully applied in order to improve culture conditions. Relatively slow rotation velocity (4 rpm) and large surface area enabling effective cell attachment are optimal parameters for cellulose production. Physical properties of BC samples synthesized either in stationary cultures or in a horizontal fermentor revealed that cellulose from stationary cultures demonstrated a much higher value of Young's modulus, but a much lower value of water-holding capacity.**
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 189–195 doi:10.1038/sj.jim.7000303

Keywords: bacterial cellulose; *Acetobacter xylinum*; rotating disc fermentor; stationary culture; Young's modulus; *Cel*$^-$

Introduction

Bacterial cellulose (BC), a biopolymer produced by some strains of *Acetobacter*, has unique physical and chemical properties. These useful characteristic properties result from specific and unique BC ultrastructure, which is particularly characterized by a net of ultrafine cellulose ribbons [21,28].

Several techniques for BC production have been reported [5,6,22,28], some of which demonstrate a potential tool for economic and commercial BC production: stationary culture (in plastic trays), agitated culture (jar fermentors), cultivation in horizontal fermentors or cultivation in internal loop airlift reactors [4,14,15,23].

The choice of a cultivation technique is dependent on further biopolymer commercial destination, considering that cellulose ultrastructure and its physical and mechanical properties are strictly influenced by the culture method [1,10]. In stationary culture conditions, a thick, gelatinous membrane of BC is accumulated on the surface of a culture medium, whereas under agitated culture conditions, cellulose can be produced in the form of a fibrous suspension, irregular masses, pellets or spheres [9,21,28]. While stationary culture has been widely investigated and applied for production of some successful commercial cellulose products (*nata de coco*, transducer diaphragms, wound care dressing materials), agitated culture is considered more suitable for the commercial production of BC mainly due to the higher production rates that potentially can be achieved [4,17,20,30]. However, cellulose production in fermentors with continuous agitation and aeration encounters many problems, including spontaneous appearance of *Cel*$^-$ mutants (cellulose nonproducers), which contributes to a decline in polymer synthesis [21]. Recent investigations showed that in agitated cultures, high oxygen supply and high volumetric agitation power are required for increase of BC productivity [16]. Other factors such as agitator configuration, effects of oxygen and carbon dioxide pressures on BC productivity have been investigated [15,16,32].

Efforts to apply other types of bioreactors for efficient cellulose production have been undertaken. Chao *et al* [6] successfully used an airlift reactor provided with oxygen-enriched gas supply to improve the oxygen transfer rate and obtain a high cellulose production rate. Static continuous culture in trays — in which the pellicle synthesized on the surface is picked up, passed through the bath in order to kill the cells and set on the winding roller — was recently reported by Sakairi *et al* [22]. Cellulose production in horizontal fermentors, which is considered to be a combination of stationary and agitated cultures, has also revealed promising results [5,23].

In our research, we examined the usefulness of *Acetobacter xylinum* E_{25} for efficient production of BC in selected culture conditions. Our studies started with characterization of *A. xylinum* E_{25} with regard to its tendency to form spontaneous *Cel*$^-$ mutants under different culture conditions. We investigated a process of BC production in stationary cultures, which were the most suitable method for cultivation of our strain. Horizontal bioreactors equipped with either rotating discs or rollers have also been successfully applied in order to improve culture conditions. Simultaneously, some characteristic physical and mechanical properties of the synthesized polymer have been determined.

Materials and methods

Microorganism

A. xylinum E_{25} (recently reclassified as *Gluconacetobacter xylinus*) [29] from the collection of the Institute of Technical Biochemistry, Technical University of Lodz, Poland, was used.

Correspondence: Dr Stanislaw Bielecki, Institute of Technical Biochemistry, Technical University of Lodz, Stefanowskiego 4/10, Lodz 90-924, Poland
Received 1 March 2002; accepted 18 July 2002

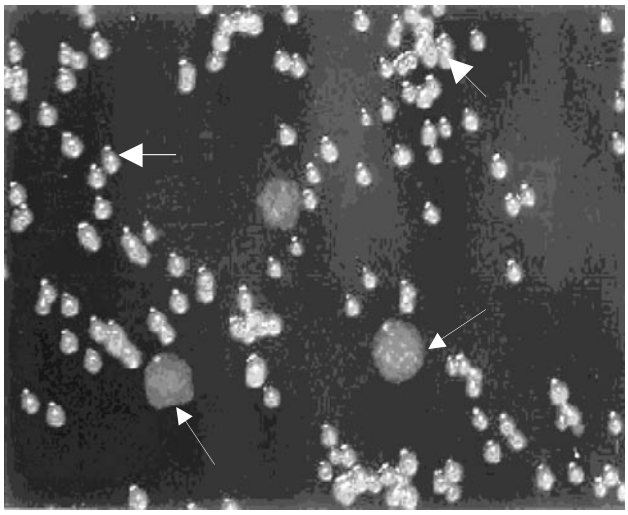

Figure 1 Morphology of *A. xylinum* E_{25} colonies grown on SH agar. Thick arrows indicate colonies of cellulose producers (Cel^+) and thin arrows indicate colonies of cellulose nonproducers (Cel^-).

Culture medium

The Schramm and Hestrin medium [11] with initial pH adjusted to 5.7 or its modifications, as well as Yamanaka medium [30] or its modifications (described in the text), were used unless otherwise specified.

Culture conditions

Preinoculum for all experiments was prepared by transferring a single *Acetobacter* Cel^+ colony grown on SH agar medium into a 50-ml Erlenmeyer flask filled with liquid SH medium. After 48–64 h of cultivation at 30°C, the cellulose pellicle formed on the surface of the culture broth was either squeezed or vigorously shaken in order to remove active cells embedded in the cellulose membrane. Ten milliliters of the cell suspension was introduced into a 500-ml Erlenmeyer flask containing 100 ml of a fresh SH medium. The culture was carried out statically for 48 h and the cell suspension derived from the synthesized cellulose pellicle was used as the inoculum for further cultures.

All cultures were grown at 30°C. The stationary cultures in either plastic trays ($0.25\times0.17\times0.08$ m) or in Erlenmeyer flasks filled with different volumes of the medium lasted for 7 days. Cultures in horizontal bioreactors equipped with discs or a roller were carried out in two fermentors having different volumes: 11 and 2l, respectively, for 7 days. Agitated cultures were carried out in 500-ml flasks on a rotary shaker at 90 rpm. The synthesized cellulose was harvested, purified by boiling it in 1% NaOH for 2 h, treated with 5% acetic acid and finally thoroughly washed in tap water until the product became transparent.

Table 1 The effect of culture medium composition on the synthesis of BC

Medium	7-day culture		14-day culture	
	pH	BC yield [g/l]	pH	BC yield [g/l]
SH	3.68	0.47	3.22	0.52
SHE	3.47	3.10	3.12	3.31
Y	4.13	0.35	3.61	0.48
YE	3.65	2.88	3.28	3.27
YM	–	–	4.98	5.05
YME	–	–	4.78	5.35

SH — Schramm and Hestrin medium enriched with 0.05% $MgSO_4$ (wt/vol); SHE — SH medium with 1% ethanol (vol/vol); Y — Yamanaka medium; YE — Yamanaka medium with 1% ethanol (vol/vol); YM — mathematically optimized Yamanaka medium; YME — mathematically optimized Yamanaka medium with 1% ethanol (vol/vol).

Stability studies of A. xylinum E_{25}

The stability of the strain has been studied using a modified method described by Ben-Bassat [1], involving serial transfers in stationary and agitated cultures. Under stationary conditions, the cultures were incubated for 48 h after which a cell suspension from the vigorously shaken cellulose pellicle was used to inoculate fresh medium in the successive static culture. This process has been repeated for four subsequent transfers. The cell suspension from each transfer was diluted, spread on SH agar and incubated for 6 days at 30°C. After that time, the appearance of *Acetobacter* colonies was examined at a magnification of 12× in order to select and count Cel^- mutants. The same procedure has been applied for agitated cultures. Based on the morphological differences, the stability of the strain was determined.

Determination of water-holding capacity (WHC)

WHC was determined by the modified method described elsewhere [28]. Cellulose samples were centrifuged for 15 min at various centrifugal forces. The volume of remaining liquid was measured. WHC was described as the ratio of the liquid volume to the cellulose dry weight.

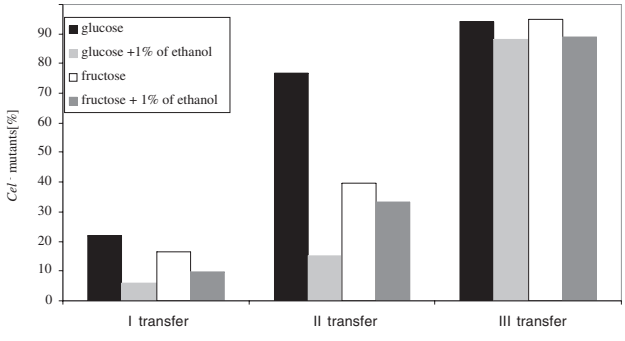

Figure 2 Effect of medium composition on *A. xylinum* E_{25} stability.

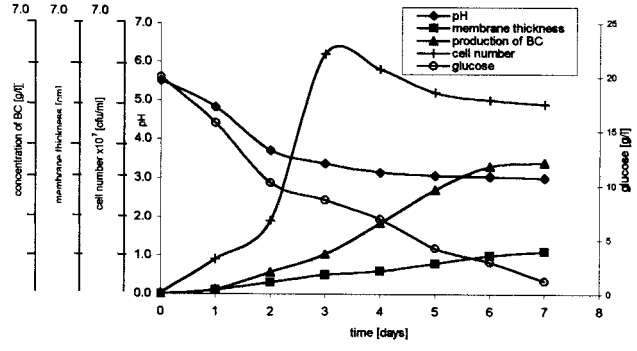

Figure 3 Dynamics of cellulose synthesis by *A. xylinum* E_{25} under stationary culture conditions.

Table 2 Influence of S/V coefficient on the cellulose production yield (plastic tray, 0.25×0.17×0.08 m)

Medium volume, V [ml]	Thickness of medium layer [cm]	Surface, S [cm^2]	S/V [cm^{-1}]	Membrane thickness [cm]	Wet membrane mass [g]	Dry membrane mass [g]	BC yield [g/l]
200	0.47	425	2.13	–	–	0.54	2.7
400	0.94	425	1.06	0.8	203.4	0.96	3.1
600	1.41	425	0.71	1.1	–	2.1	3.5
800	1.88	425	0.53	1.2	440.64	2.4	3.0
1200	2.82	425	0.35	1.25	522.13	2.86	2.4
1600	3.74	425	0.27	1.2	621.5	3.13	1.93

Mechanical testing

Mechanical properties (tensile strength, Young's modulus) of dried cellulose samples have been measured using a ZWICK 1435-type mechanical tester.

TEM and SEM

Fixed and dehydrated samples were either freeze-dried or critical point-dried (Samdri-790; Tousimis Research) and then coated with gold (30800; Ladd Research Industries). A Hitachi S-4500 field emission scanning electron microscope at 10 or 15 kV was used for sample examination. TEM observations were performed using a Philips 420 transmission electron microscope at 100 kV.

X-ray diffraction

X-ray diffraction spectra were recorded using an HZG-4 diffractometer at 30 V and 25 mA. Scans were performed over the 5–40° 2**θ** range using step 0.1° in width. The analysis of the diffractogram was carried out using the method of Hindeleh and Johnson [12]. The crystallinity index was estimated by dividing the area of the resolved crystalline peaks by the total area of the diffraction profile for 5–40°.

Results and discussion

Stability studies of A. xylinum under different culture conditions

The major obstacle encountered in agitated and highly aerated cultures of *A. xylinum* is the tendency of cellulose-producing strains to revert to noncellulose-producing mutants (*Cel*$^-$ mutants), which contributes to a decline in BC production [2,9,21].

There are some hypotheses which state that one of the possible reasons responsible for the inactivation of the gene coding cellulose synthase is transposable elements capable of moving to a new site in the genome [7]. Different culture conditions are the selective factor that affects formation of these *Cel*$^-$ mutants. As early as in 1954, Hestrin and Schramm [11] reported and described in detail a spontaneous occurrence of *Cel*$^-$ mutants in agitated cultures. They isolated morphologically different types of *Acetobacter* colonies, which displayed different abilities to synthesize cellulose [29]: *Cel*$^+$, fully able to synthesize cellulose (round, gelatinous and markedly convex colonies); *Cel*$^-$, cellulose-nonproducing mutants (generally flat and dull colonies) with or without the ability for reversion to *Cel*$^+$ forms.

As reported by a number of researchers, under stationary culture conditions that do not allow for a uniform medium aeration, *Cel*$^+$ colonies dominate. This phenomenon can be explained in terms of *Acetobacter* cells' behavior upon culturing under different conditions: in static conditions, cellulose-synthesizing cells move towards the oxygen-rich medium–air interface, where they form a gelatinous membrane that limits access of oxygen into the lower parts of the culture. In agitated cultures that provide a sufficient and uniform aeration, intensive cell growth is preferred instead of polymer synthesis, which results in *Cel*$^-$ domination in the whole culture (Figure 1).

We examined the influence of different medium compositions on occurrence of *A. xylinum* E_{25} mutants both in stationary and agitated culture conditions.

We have shown that in stationary cultures, the type of carbon source and presence or absence of ethanol do not have any significant impact on *Cel*$^-$ mutants' occurrence. In the whole stationary culture, *Cel*$^-$ forms were not observed. In contrast, in agitated cultures, *Cel*$^-$ mutants occurred immediately after the first culture transfer and their numbers increased continuously during subsequent transfers, reaching a maximum value of 80% after the third transfer.

Based on the results from agitated cultures, a general conclusion can be drawn that mutation frequency strictly depends on the culture medium composition. Figure 2 clearly shows that the

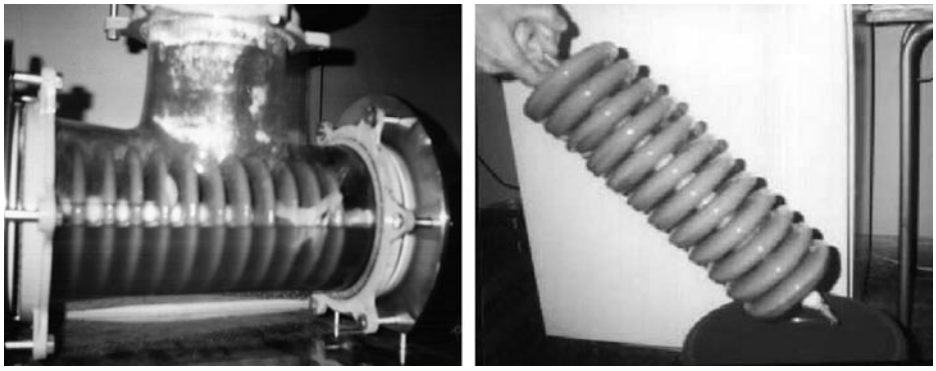

Figure 4 Biosynthesis of BC in the RDF. (a) Cultivation in RDF (b) BC attached to the discs after 7 days of culture.

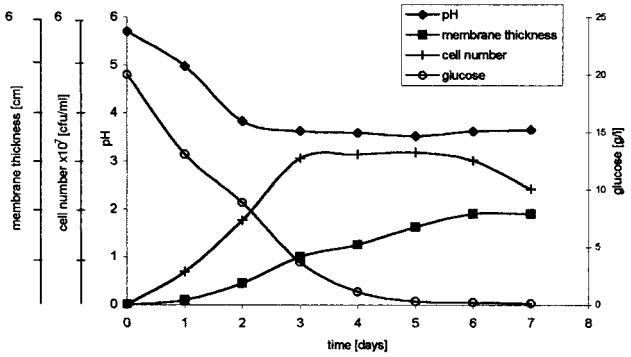

Figure 5 Dynamics of cellulose synthesis in the RDF.

maximum amount of *Cel*$^-$ mutants was observed in the original SH medium, without ethanol. A much smaller number of *Cel*$^-$ mutants appeared in the medium containing fructose as carbon source (about 35–40% after the second transfer) and in the medium containing glucose enriched with ethanol (15% after the second transfer). Furthermore, in the medium containing glucose without ethanol, the number of *Cel*$^-$ mutants reached the largest value of almost 80% of the *Acetobacter* population.

In order to determine the capability of *Acetobacter Cel*$^-$ mutants for spontaneous reversion into *Cel*$^+$ forms, we examined the cell suspension from each of the different types of cultures. Single characteristic *Cel*$^-$ colonies have been isolated from SH agar medium, transferred into fresh liquid SH medium and allowed to grow statically for 7 days. In the case of a successful reversion, the cellulose pellicle has been observed on the surface of a culture broth after a few days. The cellulose membrane was not formed in the case of nonreverting *Cel*$^-$ mutants. About 20% of all *Cel*$^-$ colonies displayed the ability for reversion to active cellulose producers; however, an increase in a number of transfers coincided with an enhancement of a number of *Cel*$^-$ mutants that could not revert to *Cel*$^+$ forms.

Stationary culture

Our stability studies proved that *A. xylinum* E_{25} is suitable for BC production in stationary cultures, whereas agitated cultures promote formation of *Cel*$^-$ mutants and result in a significant reduction of BC synthesis. These findings confirmed our preliminary studies focused on BC synthesis in stirred and aerated bioreactors using *A. xylinum* E_{25} (data not shown), which still have to be investigated in order to find the optimal conditions. Thus, our further efforts have been aimed at aspects of BC production in stationary cultures.

Table 3 Effect of rotation speed on BC synthesis

Rotation speed [rpm]	BC yield [g/l]	Time of cultivation [days]						
		1	2	3	4	5	6	7
		Cellulose membrane thickness [cm]						
4	2.82	0	0.45	1.0	1.25	1.62	1.90	1.90
6	2.10	0	0.45	0.95	1.20	1.50	1.60	1.60
8	1.89	0	0.20	0.73	1.12	1.70	1.75	1.75
10	1.70	0	0.74	1.22	1.55	1.65	1.77	1.77
12	1.72	0	0.78	1.30	1.47	–	–	–
16	1.78	0	1.0	1.45	1.65	1.75	2.25	2.25

Table 4 Effect of different surface areas (number of rotating discs) on BC production

Number of discs	Distance between the discs [cm]	Time of cultivation [days]							BC yield [g/l]
		1	2	3	4	5	6	7	
		Cellulose membrane thickness [cm]							
11	1.2	0	0.35	0.5	0.7	0.9	1.0	1.0	2.3
12	1	0	0.40	0.65	0.75	1.0	1.1	1.1	2.65
18	0.8	0	0.35	0.45	0.85	0.9	1.05	1.1	3.0
21	0.6	0	0.30	0.55	0.60	0.85	1.1	–	3.1
24	0.4	0	0.25	0.40	0.55	–	–	–	3.3
Roller	–	–	–	–	–	–	–	–	1.96

The composition of the nutrient medium as well as the strain activity are the fundamental factors affecting BC production and the profitability of the biotechnological process. The model SH medium [11], containing glucose as a carbon source and yeast extract and bactopeptone as nitrogen sources, is most commonly used in studies on BC synthesis by *Acetobacter* strains. In several studies on factors affecting BC production yield, not only glucose but also other carbon sources — like numerous mono-, di- and polysaccharides, as well as some organic compounds (i.e., glycerol) metabolized *via* gluconeogenesis pathway — were used. It is an ability to produce a required hydrolase by the bacterial strain which enables it to use oligo- and polysaccharides as carbon sources. Otherwise, enzymatic hydrolyzates of these substrates are applied [24].

We used a medium based on that developed by Hestrin and Schramm, which according to our experiences (unpublished data) had to be modified by the addition of 1% (vol/vol) ethanol and 0.05% (vol/vol) $MgSO_4 \cdot 7H_2O$ to provide good growth of *A. xylinum* E_{25}. It has been also reported by others that the presence of ethanol in the culture medium significantly enhances cell concentration and production rate [19]. Ethanol functions as an additional energy source for ATP generation, allowing glucose to be used only for BC synthesis [24]. We also used Yamanaka medium [30] containing sucrose as a carbon source and found that the addition of 1% ethanol was also highly profitable. Table 1 shows the yield of cellulose production and the pH of the culture medium after 7 and 14 days of cultivation. Our modification of Yamanaka medium was based on the mathematical optimization procedure, in which four compounds (sucrose, fructose, yeast extract and $(NH_4)_2SO_4$) have been taken into account [10]. *A. xylinum* E_{25} synthesizes a comparable amount of cellulose while growing on SHE and YE medium. However, from an economical point, YE medium is

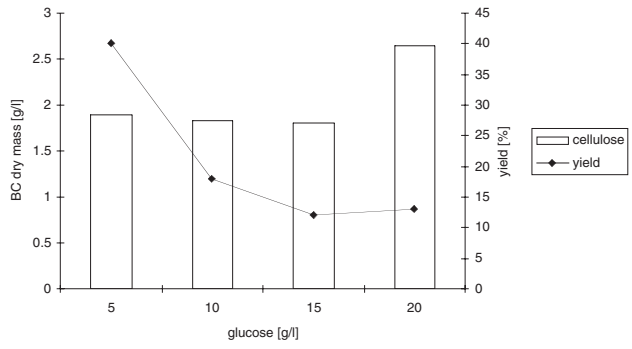

Figure 6 Effect of different initial glucose concentrations on BC synthesis yield.

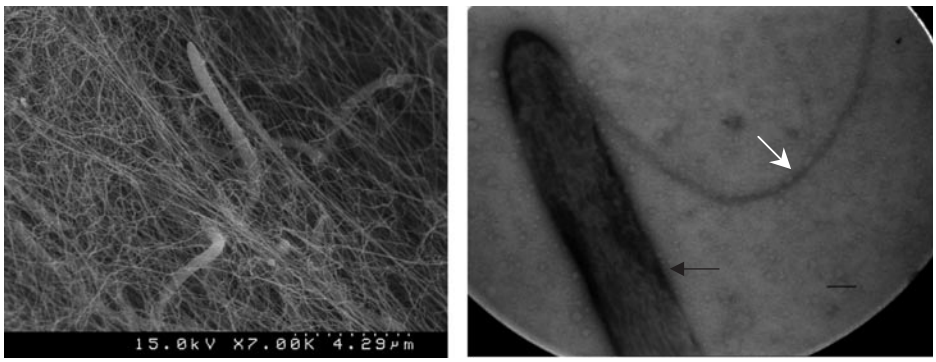

Figure 7 SEM and TEM micrographs of BC. (a) BC structure (b) *A. xylinum* cell synthesizing cellulose ribbon. Black arrow indicates *Acetobacter* cell and white arrow indicates cellulose ribbon; scale bar, 0.2 μm. Images were captured using *A. xylinum* NQ5 (ATCC 53582) strain from the collection of the Section of Molecular Genetics and Microbiology, University of Texas at Austin, USA.

cheaper and seems to be more convenient for BC production. Better results can be obtained using YME medium containing 10 times more yeast extract (5% wt/vol) and 4% sucrose (wt/vol). Only such a high concentration of yeast extract provides an amount of cellulose comparable to that obtained from a medium without ethanol.

Figure 3 demonstrates a typical time course of BC synthesis in a stationary culture. According to the data shown in Figure 3, it can be concluded that the yield of biosynthesis of cellulose increased simultaneously with the rise of cell numbers and is maintained in the stationary phase until the sixth or seventh day. The main enhancement of cellulose mass occurred in the fourth and fifth days despite a significant decrease in pH. In our case, the cells utilized approximately 50% of glucose just after the second day of the culture. A large part of this glucose was metabolized to keto- or gluconic acids [25], which resulted in the pH value decreasing to 3.5. The amount of cellulose produced during that time was estimated at 0.5 g/l.

Optimal aeration of the culture medium determined by the surface/volume (S/V) ratio is also an important factor affecting BC synthesis in stationary cultures. The optimal S/V value for the given culture conditions has been determined to be 2.2 cm^{-1} [13]. In our studies carried out in plastic trays with a surface area of 425 cm^2, the S/V ratio of 0.71 cm^{-1} gave the highest yield of cellulose (Table 2).

Other important factors can significantly affect BC synthesis. Watanabe and Yamanaka [27] examined the effect of oxygen tension in the gaseous phase on production and physical properties of BC formed in static cultures and stated that cellulose production was much higher at the oxygen tensions of 10% and 15% than under atmospheric conditions. Verschuren *et al* [26] investigated the development of oxygen concentration gradients and the effective diffusion coefficient, D_e, of oxygen in the cellulose pellicle produced under static conditions. A high oxygen flux of 9.1×10^{-6} mol/m^2 s was obtained at pH 4.0 while the effective diffusion coefficient of oxygen equal to 1.4×10^{-9} m^2/s was obtained under all conditions investigated. The penetration depth analysis clearly showed that during the early part of the culture, oxygen was a limiting factor for BC production, but at the latter stage of cultivation, carbon source also became limiting.

Table 5 Physical properties of BC produced under different conditions

Sample	Young's modulus [GPa]	Tensile strength [MPa]	Crystallinity index [%]
Stationary	2.7	92.0	50
RDF	0.3	22.9	–

BC production in horizontal fermentors

The control of BC synthesis in stationary cultures is very difficult since the pellicle limits access to the liquid medium, as well as causes some problems with proper oxygen supply required for growth of *Acetobacter* cells. In order to overcome these obstacles and to facilitate a nutrient medium supply as well as to create a better environment for growth, we used a combination of stationary and agitated culture in horizontal fermentors.

A general rule in the construction of such bioreactors is to create optimal conditions for attachment of cells on the surface of rotating rollers or discs. A specific biofilm of cells and polymer is temporarily dipped in the liquid medium during cultivation. A process of BC biosynthesis in such bioreactors displays many advantages. Cellulose in the form of a membrane can be obtained and it also allows for direct modification of BC product during synthesis (addition of different medium components or modifying agents) as well as direct measurement of different culture parameters (pH, oxygen, cell number). Different types of cultivation (batch, fed-batch or continuous) are possible using horizontal fermentors.

In our study, two different types of horizontal fermentors equipped either with plastic discs (RDF, rotating disc fermentor) (Figure 4) or with rollers, with a total volume of 2 or 11 l,

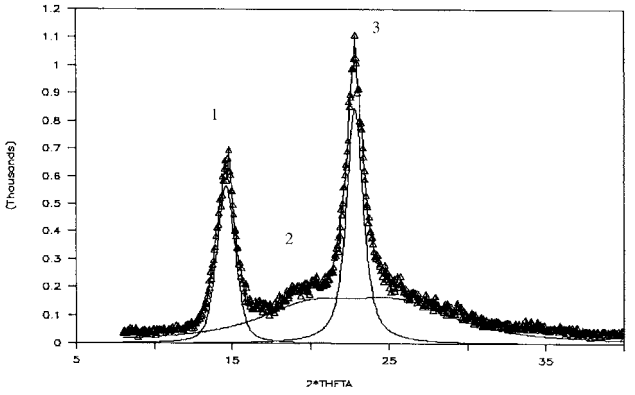

Figure 8 X-ray diffraction patterns obtained from BC samples synthesized in stationary culture conditions. Three typical diffraction peaks in the region of 10–25° are labeled as 1, 2 and 3.

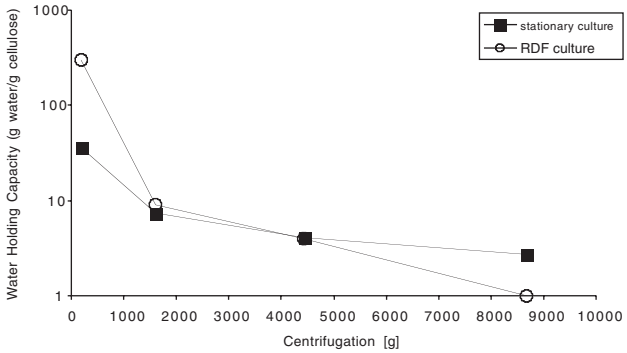

Figure 9 WHC of different BC samples.

respectively, have been used. In both cases, polymer was attached and accumulated on the rough surface of rollers or discs, which were rotated along the long axis and were temporarily dipped in the liquid nutrient medium.

The typical dynamics of cellulose synthesis in the RDF is shown in Figure 5.

The data reveal many similarities between the time course of BC synthesis in RDF and in a typical stationary culture carried out in flasks or trays. Some differences can be related to the membrane thickness, which was slightly higher in the case of culture in RDF. Other parameters, such as cell numbers, pH and glucose utilization, are similar.

Experiments on an effect of different rotation speeds and different numbers of discs (different surfaces for biofilm formation) in RDF revealed the optimal parameters for BC production. Rather slow rotation gave higher yields of BC, as shown in Table 3.

At a higher rotation speed, cellulose membranes that accumulated on the surface of the discs were much thicker despite the fact that the total mass of synthesized cellulose was lower.

To elucidate this phenomenon, a method enabling measurement of the thickness of the liquid layer, which is accumulated on the surface of the discs in cultures at different rotation speeds, was applied. An increased velocity of rotation brought about a larger amount of liquid adsorbed on the surface of the discs (data not shown). Considering these findings, we can assume that the thicker BC membranes synthesized at higher rotation speeds are the result of a much higher degree of cellulose hydration. The liquid layer on the surface of the disc is dependent on disc diameter and material used as well as on the depth of the disc immersion in the culture medium. Our experiments, which were concentrated on an influence of the surface area on BC synthesis, showed that an increase in the number of discs (which correspond to the larger surface area) enhances BC production (Table 4). We found that 24 discs (surface area of 0.206 m^2) was the optimal number in our experiments. Such a high surface area accessible for cells improved cellulose synthesis. However, application of a higher number of discs drastically decreased the distances between them and, in consequence, the adjacent membranes started to stick together. This was a reason for synthesis of irregular cellulose mass instead of separate cellulose membranes. We also applied a roller instead of the series of discs, but the production yield declined, indicating that surface area is one of the critical factors influencing cultivation in RDF. BC in the form of a tube was obtained using a horizontal fermentor equipped with roller. Because the size of such a roller can be easily modified, BC tubes with different diameters might be synthesized in this way. A successful medical application of the cellulose tubes produced under stationary conditions as artificial blood vessels has been reported [31].

We applied different initial glucose concentrations in standard SH medium in RDF and we found that a lower concentration of glucose was more effective in BC synthesis and gave a better production yield. Figure 6 shows that, whereas the highest production of BC and the thickest cellulose membranes were observed at the concentration of 20 g/l, the highest dry mass of BC coincided with the concentration of 5 g/l. This fact observed also by Bungay and Serafica [5] is of a great importance due to the high BC production costs in RDF.

BC structure and its physical properties

A structure of BC can be described as an ultrafine net built of entangled cellulose ribbons. Figure 7a and b shows a scanning electron micrograph of BC obtained under agitated culture conditions and a TEM micrograph of an *Acetobacter* cell during BC ribbon synthesis, respectively. The ultrastructure of such thin units — about 3.2 nm (thickness)×133 nm (width) — is a critical factor that determines the unique properties of reticulated BC [3]. A successful application of this unique material has been shown in our previous report on evaluation of the usefulness of BC membranes as a wound dressing material [18].

A typical X-ray diffractogram obtained from our BC sample demonstrates three characteristic clearly resolved peaks. The crystallinity index of almost 50% has been estimated based on that diffractogram. As it has been previously reported, structural differences between BC from stationary (SBC) and agitated cultures (ABC) can be readily noticed [8,28].

ABC is characterized by a much more disordered and denser structure with curved and overlapping cellulose ribbons. Also its microfibrils size is slightly smaller than that of SBC. Because of this unique structural characteristic, the BC displays excellent mechanical and physical properties (Table 5, Figure 8).

We compared cellulose samples synthesized either in stationary culture conditions or in the horizontal fermentor equipped with a roller. Our mechanical testing results clearly indicate that SBC displays a much higher Young's modulus and much higher tensile strength. This fact stays in a close relation with BC structures synthesized under different culture conditions. A much more relaxed and disordered BC structure from the horizontal fermentor is a result of external forces during rotation, which cause disturbances in the whole process of fibril crystallization [28]. This is supported by results obtained from WHC measurements, which show that BC from a horizontal fermentor is able to hold almost five times more water than BC obtained under stationary conditions (Figure 9). An increase in centrifugal forces drastically decreased the WHC values. While the WHC of BC samples from static culture seems to level off at $4500\times g$, the WHC of cellulose from RDF decreased continuously to almost zero at $8600\times g$. This corresponds to structural differences between cellulose samples.

Acknowledgements

The authors are most grateful to Prof. R.M. Brown, Jr. and Dr. D. Romanovicz from the Section of Molecular Genetics and Microbiology, University of Texas at Austin, USA, for their help in

performing SEM and TEM observations. The authors thank Dr. H. Kalinowska for careful reading of this manuscript.

References

1 Ben-Bassat A. 1992. Reticulated Cellulose Product, Sheets Formed Therefrom, Methods and Microorganisms for their Production. EP 0228779B1, Weyershaeuser Company, Tacoma, Washington 98477 (US).
2 Bielecki S, A Krystynowicz, M Turkiewicz and H Kalinowska. 2001. Bacterial cellulose. In: Steinbuchel A (Ed), Biopolymers: Vol. 7. Polysaccharides I. Wiley-VCH Verlag GmbH, Munster, Germany, 37–90.
3 Brown RM Jr, JHM Willison and CL Richardson. 1976. Cellulose biosynthesis in *Acetobacter xylinum*: visualization of the site of synthesis and direct measurement of the *in vivo* process. *Proc Natl Acad Sci USA* 73: 4565–4569.
4 Budhiono A, B Rosidi, H Taher and M Iguchi. 1999. Kinetic aspects of bacterial cellulose formation in *nata de coco* culture system. *Carbohydr Polym* 40: 137–143.
5 Bungay HR and G Serafica. 1997. Production of Microbial Cellulose Using a Rotating Disc Film Bioreactor. PN WO 97/05271, Rensselaer Polytechnic Institute, Troy, NY, pp. 12180-3590 (US).
6 Chao Y, T Ishida, Y Sugano and M Shoda. 2000. Bacterial cellulose production by *Acetobacter xylinum* in a 50 L internal-loop airlift reactor. *Biotechnol Bioeng* 68(3): 345–352.
7 Coucheron DH. 1991. An *Acetobacter xylinum* insertion sequence element associated with inactivation of cellulose production. *J Bacteriol* 173(18): 5723–5731.
8 Czaja W, D Romanovicz and RM Brown Jr. 2002. Structural Investigation of Cellulose Produced in Stationary and Agitated Culture by *Acetobacter* NQ5 Strain. (unpublished data).
9 Dudman WF. 1960. Cellulose production by *Acetobacter* strains in submerged culture. *J Gen Microbiol* 22: 25–39.
10 Galas E, A Krystynowicz, L Tarabasz-Szymańska, T Pankiewicz and M Rzyska. 1999. Optimization of the production of bacterial cellulose using multivariable linear regression analysis. *Acta Biotechnol* 19: 251–260.
11 Hestrin S and M Schramm. 1954. Synthesis of cellulose by *Acetobacter xylinum*: II. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. *Biochem J* 58: 345–352.
12 Hindeleh AM and DJ Johnson. 1971. The resolution of multipeak data in fibre science. *J Phys D Appl Phys* 4: 259–263.
13 Joris K, F Biliet, S Drieghe, D Brack and E Vandamme. 1990. Microbial production of β-1,4-glucan. *Meded Fac Landbouwwet-Rijksuniv Gent* 55: 1563–1566.
14 Kouda T, H Yano, F Yoshinaga, M Kaminoyama and M Kamiwano. 1996. Characterization of non-Newtonian behavior during mixing of bacterial cellulose in bioreactor. *J Ferment Bioeng* 82(4): 382–386.
15 Kouda T, H Yano and F Yoshinaga. 1997a. Effect of agitator configuration on bacterial cellulose productivity in aerated and agitated culture. *J Ferment Bioeng* 83(4): 371–376.
16 Kouda T, T Naritomi, H Yano and F Yoshinaga. 1997b. Effects of oxygen and carbon dioxide pressures on bacterial cellulose production by *Acetobacter* in aerated and agitated culture. *J Ferment Bioeng* 84: 124–127.
17 Kouda T, T Naritomi, H Yano and F Yoshinaga. 1998. Inhibitory effect of carbon dioxide on bacterial cellulose production by *Acetobacter* in agitated culture. *J Ferment Bioeng* 85(3): 318–321.
18 Krystynowicz A, W Czaja, L Pomorski, M Kolodziejczyk and S Bielecki. 2000. The evaluation of usefulness of microbial cellulose as wound dressing material. 14th Forum for Applied Biotechnology, Gent, Belgium, Proceedings Part I, Meded Fac Landbouwwet-Rijksuniv Gent, 213–220.
19 Naritomi T, T Kouda, H Yano and F Yoshinaga. 1998. Effect of ethanol on bacterial cellulose production from fructose in continuous culture. *J Ferment Bioeng* 85(6): 598–603.
20 Ring D, W Nashed and T Dow. 1986. Liquid Loaded Pad for Medical Applications. US Patent 4,588,400.
21 Ross P, R Mayer and M Benziman. 1991. Cellulose biosynthesis and function in bacteria. *Microbiol Rev* 55(1): 35–58.
22 Sakairi N, H Asano, M Ogawa, N Nishi and S Tokura. 1998. A method for direct harvest of bacterial cellulose filaments during continuous cultivation of *Acetobacter xylinum*. *Carbohydr Polym* 35: 233–237.
23 Sattler K and S Fiedler. 1990. Production and application of bacterial cellulose: II. Cultivation in a rotating drum fermentor. *Zentralbl Microbiol* 145: 247–252 (in German).
24 Seto H, T Tsuchida and F Yoshinaga. 1995. Production of Bacterial Cellulose. JP07184677A. Bio-Polymer Research, Japan.
25 Vandamme EJ, S De Baets, A Vanbaelen, K Joris and P De Wulf. 1998. Improved production of bacterial cellulose and its application potential. *Polym Degrad Stab* 59: 93–99.
26 Verschuren P, T Cardona, M Nout, K De Gooijer and J Van den Heuvel. 2000. Location and limitation of cellulose production by *Acetobacter xylinum* established from oxygen profiles. *J Biosci Bioeng* 89(5): 414–419.
27 Watanabe K and S Yamanaka. 1995. Effects of oxygen tension in the gaseous phase on production and physical properties of bacterial cellulose formed under static culture conditions. *Biosci Biotechnol Biochem* 59(1): 65–68.
28 Watanabe K, M Tabuchi, Y Morinaga and F Yoshinaga. 1998. Structural features and properties of bacterial cellulose produced in agitated culture. *Cellulose* 5: 187–200.
29 Yamada Y. 2000. Transfer *Acetobacter oboediens* and *A. intermedius* to the genus *Gluconacetobacter* as *G. oboediens comb. nov.* and *G. intermedius comb. nov. Int J Syst Evol Microbiol* 50: 225–227.
30 Yamanaka S, K Watanabe, N Kitamura, M Iguchi, S Mitsuhashi, Y Nishi and M Uryu. 1989. The structure and mechanical properties of sheets prepared from bacterial cellulose. *J Mater Sci* 24: 3141–3145.
31 Yamanaka S, K Watanabe and Y Suzuki. 1990. Hollow Microbial Cellulose, Process for Preparation Thereof, and Artificial Blood Vessel Formed of Said Cellulose. EP 0 396 344 A2. Ajinomoto, Japan.
32 Yoshinaga F, N Tonouchi and K Watanabe. 1997. Research progress in production of bacterial cellulose by aeration and agitation culture and its application as a new industrial material. *Biosci Biotechnol Biochem* 61(2): 219–224.