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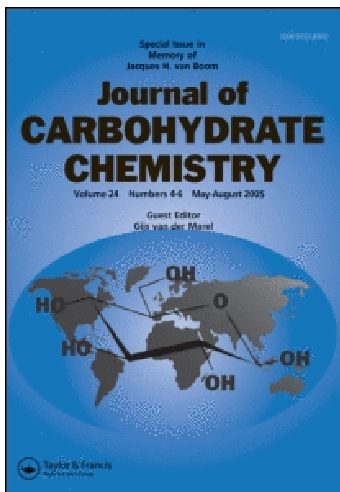

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
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The Control of Cellulose Biosynthesis by *Acetobacter Xylinum* in View of Molecular Weight and Molecular Weight Distribution Part I: Change of Molecular Weight of Bacterial Cellulose by Simple Variation of Culture Conditions

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To cite this Article Einfeldt, Lars and Klemm, Dieter(1997) 'The Control of Cellulose Biosynthesis by *Acetobacter Xylinum* in View of Molecular Weight and Molecular Weight Distribution Part I: Change of Molecular Weight of Bacterial Cellulose by Simple Variation of Culture Conditions', Journal of Carbohydrate Chemistry, 16: 4, 635 — 646
To link to this Article: DOI: 10.1080/07328309708007341
URL: http://dx.doi.org/10.1080/07328309708007341

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J. CARBOHYDRATE CHEMISTRY, 16(4&5), 635-646 (1997)

THE CONTROL OF CELLULOSE BIOSYNTHESIS BY *ACETOBACTER XYLINUM* IN VIEW OF MOLECULAR WEIGHT AND MOLECULAR WEIGHT DISTRIBUTION

Part I:

CHANGE OF MOLECULAR WEIGHT OF BACTERIAL CELLULOSE BY SIMPLE VARIATION OF CULTURE CONDITIONS [1]

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Final Form February 19, 1997

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ABSTRACT

The variation of culture conditions - such as inoculation procedures, cultivation times, subspecies of *Acetobacter xylinum* and carbon sources - lead to different bacterial cellulose products. After isolation and purification of the bacterial cellulose (BC) from the culture medium the BC was analyzed by subsequent mild trimethylsilylation to silylated cellulose followed by size-exclusion chromatography (SEC). Significant molecular weight shifts of these BC's were shown and discussed for a control of BC formation. The data of SEC from silylated BC are critically compared with a carbanilated BC.

INTRODUCTION

One fundamental problem in cellulose chemistry is the nonuniform assembling of the starting cellulosic material which is mostly produced from wood and other plant sources. Due to pulping processes it is unavoidable that the cellulose is altered in its original natural morphology, its functionality as well as its molecular weight and molecular weight

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distribution. Therefore, the demand for molecularly uniform cellulose materials for systematic investigations of chemical reactions, on self-organization or the assembly of supramolecular architectures of corresponding cellulose derivatives, directs one's attention to bacterial cellulose (BC).[2] In contrast to plant cellulose a practicable influence on cellulose formation is given through the extracellular biosynthesis of BC by *Acetobacter xylinum*.[3,4] In this respect it is our aim to study the possibilities for controlling the cellulose biosynthesis by *A. xylinum* in regard to molecular weight (MW) and molecular weight distribution (MWD) by variation of the culture conditions.

In this paper we present our first results of this ongoing research by focusing on the characterization procedures of the formed BC as follows: *(a)* trimethylsilylation of dissolved BC to get tetrahydrofuran-soluble cellulose derivatives and *(b)* SEC analysis of these products by refractive index and viscosity detection. The experiments show that BC differ in MW and MWD, depending on culture conditions and the *A. xylinum* strains used in BC biosynthesis. First trends of MW diminution of BC are shown.

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RESULTS AND DISCUSSION

Based on earlier investigations on the changes in the degree of polymerization (DP) of BC during cultivation it is known that the MW is a function of cultivation time, age of inoculation and bacteria generation time.[5] Furthermore, our own experiments on the kinetics of bacterial cellulose formation by *A. xylinum* under static culture conditions have shown remarkable influences on cellulose yield due to the dimensions of culture vessels, strains of *A. xylinum* and composition of the culture medium.[4]

In our trials a number of cultivation parameters had been kept constant to minimize such effects on bacterial cellulose formation in the different experiments. Stand cultures were carried out at 30 °C in petri-dishes (diameter: 88 mm) with 60 mL Schramm-Hestrin medium (SH-medium [6a]) containing 2% *(w/w)* carbon source generally. Furthermore we have used three different subspecies of *A. xylinum* (ATCC 10245, ATCC 23769 and AX 5) from freshly prepared stock cultures. Inoculation material was produced by three different methods:

Method A: This first method represented a usual bacteria inoculation transfer.[6]

Method B: We removed the formed cellulose network on the liquid medium surface after inoculation *method* A. After this removal we assume that with begining of cultivation most

of the cells were in the same physiological state and distributed on the medium surface ideally. In contrast to *method* A the cellulose pellicles formed now have shown regular membrane thicknesses.[7]

Method C: We inoculated with high cell mass using a slightly modified procedure described by Brown Jr.[8]

For the last two culture and inoculation methods the bacterial generation time should play a less important role by variation of culture conditions. Therefore we only varied one cultivation parameter to control the BC formation and looked at these effects in view of MW and MWD shiftings of BCs.

After cultivation the formed BC pellicles were collected from SH medium and purified to remove the nutrient medium and the immobilized bacterial cells from the cellulosic network. The purified wet cellulose pellicles were freeze-dried. These BCs showed a water content of 3% determined by Karl-Fischer-titration. It was possible to dissolve such BC in dimethylacetamide / lithium chloride directly at room temperature. Alternatively the swollen BC could also be treated by solvent exchange from water to methanol to dimethylacetamide (DMAc) obtaining cellulose soluble in DMAc/LiCl at room temperature The feature of solubility at room temperature has to be seen in view of non-hydrolysis of the glucan chains, because the described classical dissolution procedures in DMAc/LiCl by heating up to 120 °C led to cellulose degradation of about 10%.[9]

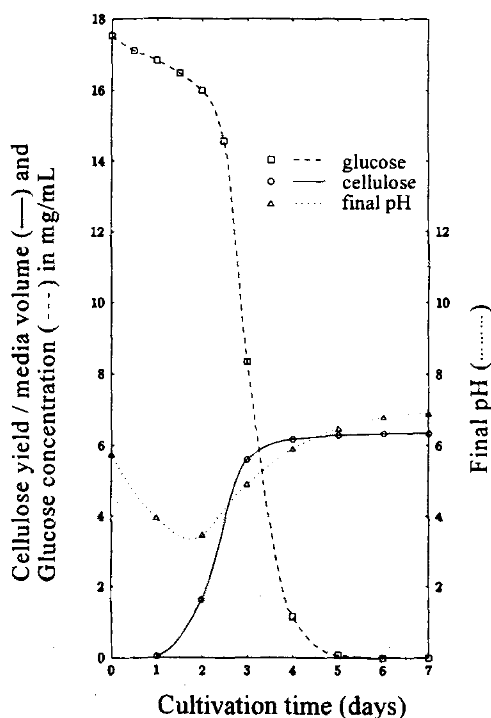

Figure 1. BC formation (total cellulose amount) by *A. xylinum* subspecies AX 5 [4] in comparison to the glucose concentration and the pH in the culture media during the cultivation of 7 days

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For the analysis of MW and MWD by size-exclusion chromatography (SEC) the BC had to be converted to an organosoluble cellulose derivative. Because of the reported high DP of BC we decided to use mild silylation procedures to get trimethylsilylcelluloses (TMS-cellulose) which were already used in SEC successfully.[10] A 10-fold *(v/w)* excess of hexamethyldisilazane and reaction temperatures around 80 °C gave TMS-celluloses with high degree of substitution ($DS_{Si} = 2.1 \pm 0.2$).

In our first experiments (*trials* 1 and 2) we investigated the influence of cultivation time by considering our specific culture conditions, the inoculation methods and the used *A. xylinum* strains. Lower MW of BC were observed at decreased cultivation times. The BC of strain ATCC 23769 shows significant differences in chain length with DP = 1800 ± 180 between 3 and 7 culture days (Fig. 2, Table 1). In principle, similar changes of MW as a function of cultivation time could be observed by the BC of strain AX 5. Between 6 and 30 culture days a MW shifting of BC of about DP = 1400 ± 140 were available (Fig. 2, Table 1). According to our knowledge these findings are due to the changed growth behaviour of the bacteria. The generation time of *Acetobacter* species have been estimated up to 8 h.[5] The main cellulose formation is carried out in the stationary phase of the bacteria, which means that after 3 - 4 days of culture the carbon source is exhausted (Fig. 1). It is necessary to determine the reasons for the significant DP differences of the BC after short and longer cultivation time, which is presently not clear. Explanations have been offered in view of enzymatic regulation of the glucan chain polymerization[5] as well as the degradation of BC by cellulase after subsequent carbon source consumption.[11] On one hand these should give lower MW at longer cultivation time on one side,[11a] but this was not observed in our attempts. On the other hand it is accepted that acetic acid bacteria - like *Acetobacter* - show no cellulase activity, a view which was questioned only recently.[11b] But in our cases the remarkable MW shifts as seen in Figures 2 and 3 could only depend on cultivation time. Otherwise the differences between ATCC 23769 and AX 5 have to be seen in different kinetics results during cultivation.

Therefore it is clear that different *A. xylinum* strains produce different BCs under the same culture conditions, inoculation volumes of corresponding stock cultures as well as cultivation time. In the next experiment (*trial* 3) we compared the formed BC from strains ATCC 10245 and AX 5 (Fig. 4, Table 1). The results are in good agreement with different reported data about DP of BC [5b] and show the necessity in specifying all cultivation conditions for critical discussions.

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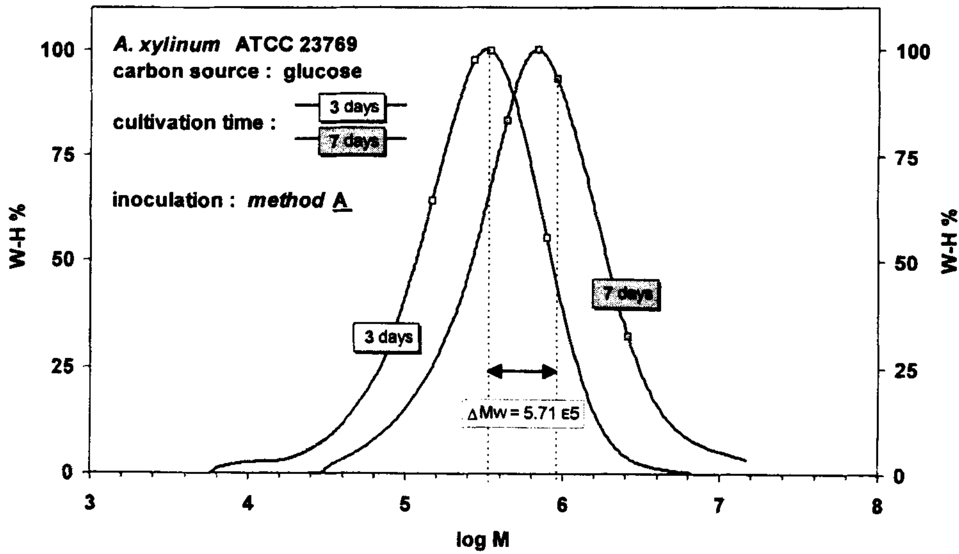

Figure 2. SEC from silylated BC of *A. xylinum* ATCC 23769: The BC were formed by different cultivation times.

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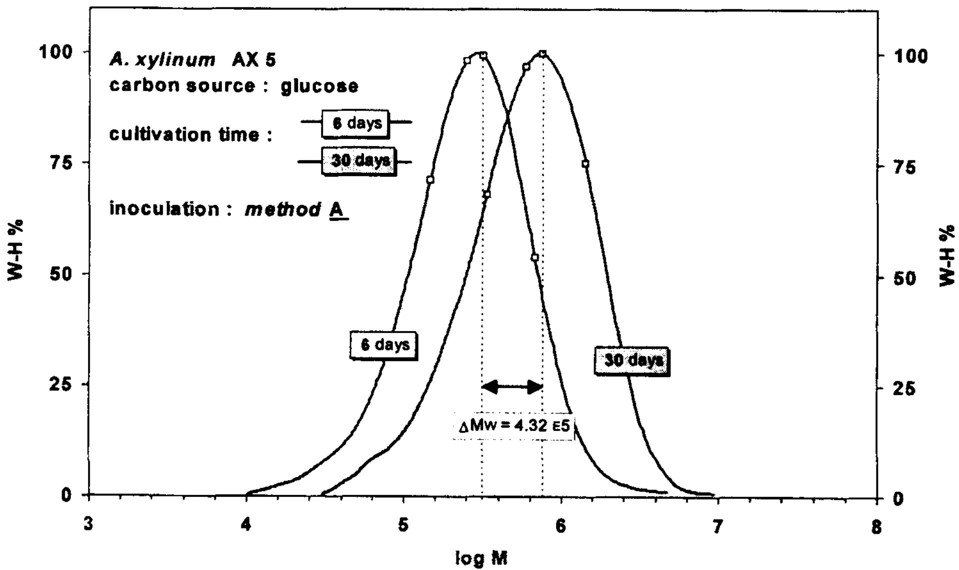

Figure 3. SEC from silylated BC of *A. xylinum* AX 5: The BC were formed by different cultivation times.

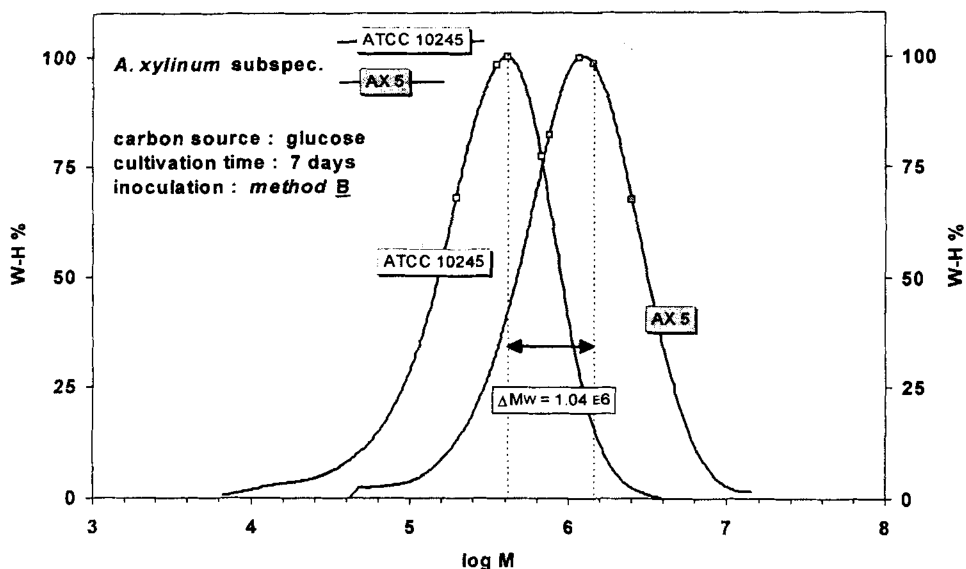

Figure 4. SEC from silylated BC of two different subspecies of *A.xylinum* by using the same culture and inoculation conditions.

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In the last experiments it was observed that by using our own *A. xylinum* subspecies AX 5 as well as carbon source mixtures such as 70 : 30 % *(w/w)* glucose : *N*-acetyl-glucosamine (GlcNac) and 70 : 30 % *(w/w)* glucose : 2-deoxyglucose (2Dglc), that it is possible to control the BC formation to lower MW (Fig. 5). Quite recently it was shown that low MW were achievable by adding 2Dglc into the liquid SH-medium on which *A. xylinum* ATCC 10821 was grown.[11] Our trial revealed significantly lower DP's: DP = 1600 ± 160 for the GlcNac medium and DP = 1400 ± 140 for the 2Dglc medium (Fig. 5, Table 1). Incorporation of these sugars into the glucan chains has been discussed.[4,12] By structure analysis it is very difficult to determine small contents of incorporated sugars into BC glucan chains in the range of maximum 4 mol% as described for GlcNac.[13]

At least some critical notes to the BC characterization should be added. As demonstrated, above only trimethylsilylated BCs (DS_{Si} = 2.1 ± 0.2) were chromatographed by SEC. Using published and developed silylation procedures silylated BC with DS_{Si} = 3.0 could not be obtained.[10] To be sure that the derivatized cellulose polymers are not exceeding the range of viscosimetrically detectable PS-standards the silylation method of BC was preferred, because our observations partly showed very high MWs of BC. The solution

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Table 1. Molecular weights (M_n, M_v, M_w, M_z) and polydispersities (M_w/M_n, M_z/M_n) of bacterial celluloses and culture conditions (strain, cultivation time, C-source, inoculation method) to get these cellulose products

FIGURES	CULTURE CONDITIONS [a]				MOLECULAR WEIGHTS				POLYDISPERSITIES	
size-exclusion chromatograms	strain of *A. xylinum*	cultivation time	carbon source	inoculation method [b]	M_n (10^5 g/mol)	M_v (10^5 g/mol)	M_w (10^5 g/mol)	M_z (10^5 g/mol)	M_w/M_n	M_z/M_n
Figure 2	ATCC 23769	**3 days**	glucose	A	*1.53*	*2.83*	*3.62*	*8.14*	*2.4*	*5.3*
	ATCC 23769	**7 days**	glucose	A	4.41	7.07	9.33	26.60	2.1	6.0
Figure 3	AX 5	**6 days**	glucose	A	*1.49*	*2.54*	*3.20*	*6.81*	*2.2*	*4.6*
	AX 5	**30 days**	glucose	A	3.37	6.01	7.52	14.50	2.2	4.3
Figure 4	**ATCC 10245**	7 days	glucose	B	*1.99*	*3.60*	*4.19*	*6.79*	*2.1*	*3.4*
	AX 5	7 days	glucose	B	7.65	11.80	14.60	25.50	1.9	3.3
Figure 5	AX 5	9 days	**GlcNac** [c]	C	*1.25*	*2.00*	*2.33*	*3.23*	*1.9*	*2.6*
	AX 5	9 days	**2Dglc** [d]	C	*1.40*	*2.43*	*2.90*	*7.50*	*2.1*	*5.4*
	AX 5	6 days	**glucose**	C	3.67	5.97	7.34	14.30	2.0	3.9

a. Varied culture conditions are written in **bold** type. b. Inoculation: *Method* A, B and C are described in MATERIALS AND METHODS. c. The carbon source is composed of 30 : 70% *(w/w)* **GlcNac** : Glc. d. The carbon source is composed of 30 : 70 % *(w/w)* **2Dglc** : Glc.

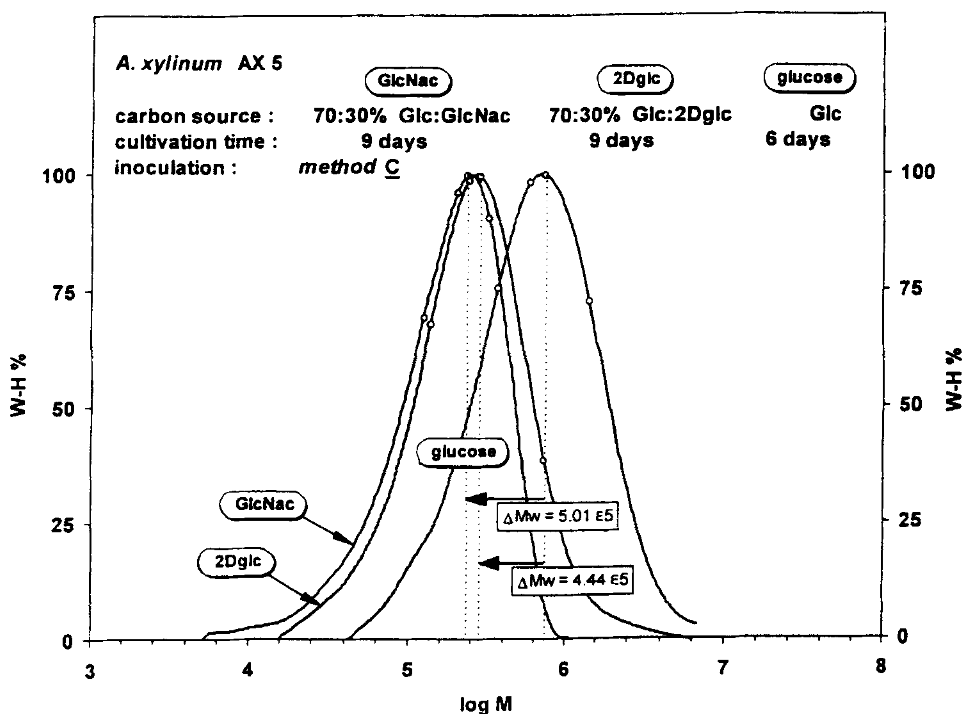

Figure 5. SEC from silylated BC of *A. xylinum* AX 5: The BC were formed on different carbon sources.

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behaviour of the synthesized TMS celluloses was not investigated. Elsewhere it is described, that incompletely substituted cellulose derivatives have different solution states of aggregation as known for synthetic linear polymers like polystyrol.[14] On the other hand, the solution behaviour of cellulose tricarbanilates is well studied and mostly preferred for SEC analysis of polysaccharides.[14,15] We compared our SEC results by dissolution of one BC sample in DMAc/LiCl, its equal division and subsequent mild carbanilation as well as silylation of each solution (Fig. 6). Probably the differences in MWs - shown as degree of polymerization (DP) in Figure 6 - and MWDs are due to the different solution behaviour of the cellulose products. It is further noted, that viscosity detections also gave a higher inaccuracy of exact determinable chain lengths at higher MWs. Therefore the measured MWs and MWDs must be considered carefully (Table 1). However in our opinion the measured results of the TMS-celluloses are reproducible.

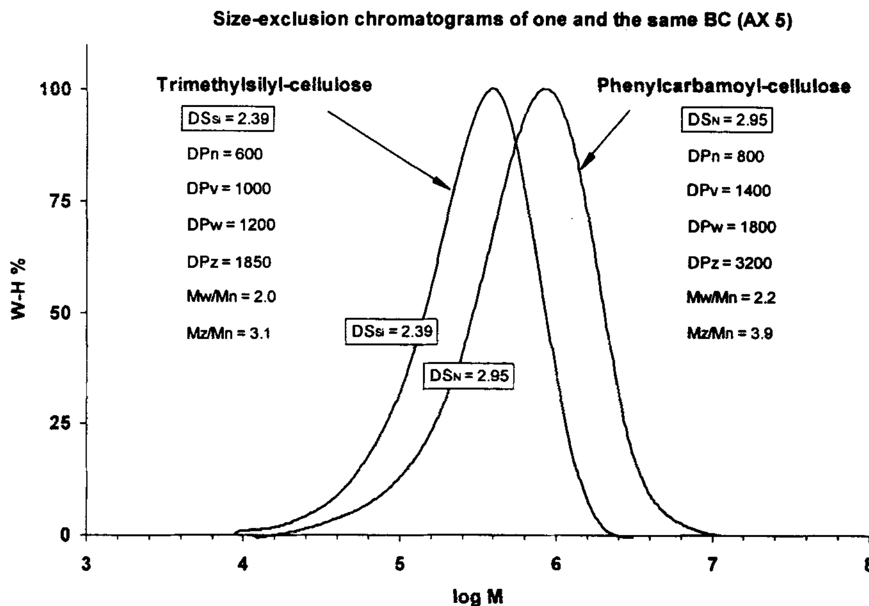

Figure 6. Comparison of trimethylsilylation and carbanilation method of BC in view of determination of MWs and MWDs by SEC

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We conclude that the significant differences in MW estimation of the compared TMS-celluloses show the controllability of cellulose biosynthesis by *A. xylinum*. The effect of different inoculations has to be seen in a decrease of cultivation time. It must be stressed that the measured MWs and polydispersities are averages over an ensemble of molecules which are formed at different times and with other variations of culture conditions (Table 1). For an understanding of the BC synthesis mechanism and controlling uniform cellulose formation, the analysis of the MWs and MWDs employing differential culture steps are necessary. Results concerning the molar mass distribution of BCs during cultivation will be reported elsewhere.

MATERIALS AND METHODS

Cultivation of *A. xylinum*:

Three different strains of *A. xylinum* were used: AX 5 (own collection), ATCC 10245 and ATCC 23769. Stand cultivation were carried out in petri-dishes (⌀ = 88 mm) with 60 mL SH-medium at 30 °C.[6] The inoculation was done by three different methods:

Method A: 1-2 ml of a stock culture was added to the 60 mL SH-medium.[6b]

Method B: The early formed soft cellulosic network was removed during bacterial log phase, that means after 1-2 days.[7]

Method C: A high cell numbering was prepared with slight modifications as described and used as inoculum to reduce the bacterial log phase.[8]

The specific culture conditions of all experiments (including the comparison with standard conditions) are summarized in Table 1.

Trial 1: Variation of cultivation time using SH medium, inoculation *method* A, and strain of ATCC 23769 (Fig. 2).

Trial 2: Same conditions as described before, but instead of ATCC 23769 the *A. xylinum* subsp. AX 5 was used (Fig. 3).

Trial 3: Variation of the *A. xylinum* subsp. (ATCC 10245 and AX 5) using the same culture conditions (SH medium, inoculation *method* B and 7 days of cultivation) (Fig. 4).

Trial 4: Variation of the carbon source in the SH medium: (a) 30 : 70 % *(w/w)* GlcNac : Glc, and (b) 30 : 70 % *(w/w)* 2Dglc : Glc (Fig. 5).

Purification of bacterial cellulose pellicles:

BC pellicles were washed with water, treated with 1% SDS- solution and washed with water again.[16] Wet pellicles were crushed by Ultraturrax®, freeze-dried and kept under P_2O_5-dried atmosphere. No nitrogen content was detectable by elemental analysis nor were any bacterial proteins by protein analysis (Lowry-method).[16]

Dissolution of bacterial cellulose in DMAc/LiCl:

Freeze-dried BC was swollen in DMAc for 1 h at room temperature. An adequate amount of dried LiCl is added to get 5-9% salt solutions and the mixture was stirred for 24 h at room temperature again. Well dried BC was dissolved by this procedure to give highly viscous 0.5% solutions. Sometimes no dissolution was achievable at room temperature. In this case only highly swollen mixtures of BC in DMAc/LiCl had existed. Any heating of such mixture at 120 °C for a few hours is also unsuccessful for dissolving the BC.[17]

Trimethylsilylation of BC solution in DMAc/LiCl:

To a 0.5% BC solution in DMAc/LiCl 6 molar equivalent per anhydroglucose unit of hexamethyldisilazane (HMDS) was added under an argon atmosphere.[10b] The stirred solution was heated at 80 °C for 24 h until the formed TMS-cellulose precipitated. If no precipitation occurred, additional amounts of HMDS were added and the reaction was

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repeated as described before. After cooling, the suspension was extracted by addition of 50% *(v/v)* hexane. The TMS-cellulose was isolated from the hexane phase by subsequent evaporation of the solvents under reduced pressure. Dissolution in tetrahydrofuran (THF) and precipitation in phosphate buffer (pH 7.0) gave TMS-celluloses purified once more by dissolving in THF and separation by precipitation in methanol. Estimation of silicon content showed $DS_{Si} = 2.1 \pm 0.2$ of TMS-celluloses.[10a] If the product showed no solubility in THF, additional silylation was carried out in THF suspension with HMDS until the TMS-cellulose was dissolved.

Carbanilation of BC solution in DMAc/LiCl:

Carbanilation of 0.5% BC solution in DMAc/LiCl was carried out by addition of an excess of phenylisocyanate and catalytical amounts of pyridine as described elsewhere.[15]

Size-exclusion chromatography (SEC):

We used a KNAUER-SEC-instrument with refractive index and viscosity detector, three utltra styrogel columns (500Å, 10^4Å and linear), THF eluation and flowrates of 1.0 mL/min at room temperature. The calibrations were carried out with polystyrene standards giving a linear function between the lowest (162 Da) and highest standard (2.750 kDa). The chromatographical data of each silylated BC sample - number average (M_n), viscosity average (M_v), weight average (M_w) and centrifugal average molecular weights (M_z) as well as the polydispersities M_w/M_n, M_z/M_n - are summarized in Table 1.

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ACKNOWLEDGEMENTS

The authors are very thankful for financial support by *Deutsche Forschungs-gemeinschaft*.

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17. The dissolution of BC in DMAc/LiCl at room temperature is very spectacular in this solvent system[9] and not known up to today. We assume that unsuccessful dissolution attempts of BC are due to small differences in the moisture content of BC.

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