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Molecular parameters of bacterial cellulose. Effect of temperature and pH biosynthesis medium

Summary — The article presents the results of studies on the effect of temperature and pH of the culture medium on molecular properties (M_w and M_n values, polydyspersity, radius of gyration) and on the productivity of biosynthesis of bacterial cellulose using gram-negative bacteria *Acetobacter xylinum*. The results prove that the temperature of the process significantly affects the properties of the product. It was found out that the optimum temperature of biosynthesis is about 30 °C whereas the pH value of the culture medium ranging from 3.8 to 7.5 only slightly affects the properties of the obtained bacterial cellulose.

Key words: bacterial cellulose parameters of biosynthesis, molecular characteristics, gel permeation chromatography.

The most important research fields of biotechnology include technological processes taking advantage of the activity of microorganisms. These processes are based, among others, on the ability of microorganisms to produce polysaccharides, *e.g.* cellulose [1, 2]. Syntheses of this type are carried out under controlled conditions independent on atmospheric ones, and resulting polysaccharides are characterised by specific properties, quite different from those of polysaccharides formed in natural conditions [3—5]. These unique features predestine the obtained polysaccharides to special applications, *e.g.* cellulose produced by bacteria, *i.e.* bacterial cellulose (BC), is successfully used to make the membranes of loudspeakers [3, 5, 6]. Moreover, by introducing various components into the culture medium, the polysaccharides can be modified what markedly widens a potential offer of microbiological synthesis products [7].

Bacterial cellulose (BC) is one of the most important polymers produced by microorganisms. In biosynthesis of BC, gram-negative bacteria *Acetobacter xylinum* appear to be the most effective ones [8—10]. BC is produced in the form of microfibriles being liberated outside the cell. Microfibriles join together forming a gel-like film on the surface of a liquid culture medium. The film, after purification and drying, resembles a paper-like sheet. Contrary to the plant cellulose, BC is chemically pure (α-cellulose content > 99.5%) and thus it needs no special purification or other additional chemical treatment.

Despite numerous and time-consuming efforts to investigate microbiological synthesis of a cellulose, many aspects of the process have not yet been explained. Therefore further experiments are continually performed to find the correlation between biosynthesis parameters and the properties of BC.

The present article describes the results obtained from the examination of the effect of temperature and pH of the medium on molecular characteristics of bacterial cellulose and on the productivity of biosynthesis process as well as purity of the product. The effect of the process duration and the composition of the culture medium have been presented earlier [11].

To examine the molecular characteristics of BC samples synthesized in various conditions, gel permeation chromatography (GPC) method has been used. Due to the application of two detectors (refractive index and viscometric ones) the method enables to obtain comprehensive information concerning all important molecular parameters of the polymer, *i.e.* number- and weight-average molecular weights (M_n and M_w), polydispersity (M_w/M_n), molecular weight distribution (*MWD*) as well as some characteristics describing the properties of BC in its diluted solutions, *i.e.* intrinsic viscosity [η] and radius of gyration of macromolecular coil (R_{gz}).

EXPERIMENTAL

Biosynthesis of bacterial cellulose

Bacterial strain

The *Acetobacter xylinum* strain produced at the Institute of Microbiology and Fermentation, Technical Uni-

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versity of Łódź, Poland was applied in the studies of cellulose biosynthesis.

Culture media

The following culture media based on Hestrin Schramm cultures were used [9]:

— Standard culture medium: glucose 2% (w/v), yeast extract 0.5% (w/v), disodium phosphate 0.27% (w/v), citric acid 0.12% (w/v), ethyl alcohol 2% (w/v); pH 6.0.

— Culture media with pH ranging from 3.8 to 7.5; pH was regulated by changing the amount of citric acid and disodium phosphate, other components as in the standard medium.

Extract of yeast is a typical ingredient of microbial media and is commercially available (Difco, USA).

Inoculum preparation

Solid components of the culture medium were dissolved in distilled water, sterilized in autoclave at 121 °C and then ethyl alcohol was added in the amount of 2% (v/v). The culture medium was inoculated by *Acetobacter xylinum* strain, *i.e.* liquid culture of *Acetobacter xylinum* was added to the culture medium in proportion 1:10 (v/v) and incubated for 3 days at 30 °C in an incubator [10].

Biosynthesis of BC by static method

The inoculum prepared this way was added to the new batch of the culture medium previously sterilized in an autoclave [in proportion 1:10 (v/v)]. The synthesis of cellulose was carried out in glass crystallizers using a static method at different temperature and culture medium pH for 7 days.

Separation of BC from culture medium

After filtration of the culture medium, the obtained cellulose film (BC) was washed first with distilled water, next with 2% NaOH aq. solution and then it was sterilized in an autoclave at 121 °C for 15 min. After sterilization, the cellulose was washed with distilled water, poured with 1% aq. solution of acetic acid and again washed with water. BC was spread to dry at 120 °C [10].

Methods

Determination of α-cellulose content

α-Cellulose content in BC samples was evaluated using gravimetric method described in [12] by determining the content of fraction insoluble in 17.5% NaOH solution.

Gel permeation chromatography

- **Preparation of BC solutions for GPC analyses**

BC samples were dissolved according to Ekmanis [13] and following further modifications of his method [14, 15] with the application of microwave heating.

The procedure described in the mentioned papers consists of four stages. Firstly cellulose is swollen in water to cause the opening of its structure — 20 mg of BC is placed in a 50 mL beaker in distilled water for 48 h. The sample is then wrung and immersed in to 10 mL of dimethylacetamid (DMAC) and next placed in a microwave oven and heated at about 160 °C for 1 min. The excess of DMAC is removed and then a new portion of 10 mL of DMAC is introduced into the vessel. The operation of DMAC exchange in the oven is repeated twice. After removing DMAC the sample is transferred into 25 mL flask containing 7.5 mL of DMAC/8% LiCl. During the first several hours swelling of a cellulose occurred in a static conditions. After that the flask was gently shaken periodically (8 hours per day) at ambient temperature (21±1 °C). Dissolving of BC in this system is completed after 2—3 days. After dissolving, the solution is diluted with DMAC to obtain finally a solution of concentration of about 0.2 mg/L in DMAC/0.5% LiCl. The solution was vigorously shaken, then heated at 80 °C for 0.5 h in order to bring about protein denaturation and possibly to dissolve precipitated macromolecules, and after 3—4 hours it was filtrated using 1 μm filters (Gelman). After the dilution the solution of BC was injected to the GPC system within 3—6 hours.

The application of microwave heating considerably shortens the time of solvent exchange in the cellulose structure in comparison with other methods [13, 16]. Microwave heating probably brings about a slight degradation of cellulose caused, however, rather by the action of high temperature (about 160 °C) than by microwaves [17].

LiCl and DMAC were carefully dried out before preparation of DMAC/0.5% LiCl and DMAC/8% LiCl solutions. LiCl was dried in a vacuum at 105 °C for 48 hours. DMAC was dried on 4Å molecular sieves.

- **Description of GPC system**

Number and weight molecular weights (M_n and M_w), polydispersity (M_w/M_n) as well as intrinsic viscosity [η] and radius of gyration (R_{gz}) of BC were determined using GPC method. The GPC system consists of DG-700 degasser (Viscotek, Houston Texas, USA), HP 1050 pump (Hewlett-Packard, Waldrom, Germany), a sample injector (Rheodyne Inc. Model 7125 Cofati Texas USA), on-line filter (2 μm), a set of three columns PLgel Mixed A (300×7.5 mm) with a guard column (Polymer Laboratories Ltd. Shropshire, UK), differential viscometric detector H502B (Viscotek) and refractive index detector HP 1047 (Hewlett-Packard). Temperatures of cells were maintained at 80 °C (viscometric detector) and at 50 °C (refractive detector). Detectors were connected in series. DMAC/0.5% LiCl was applied as an eluent. Conditions of measurements were: the temperature of column set — +80 °C, flow rate of the eluent — 0.4 mL/min, volume of the injection —150 μL. The registration of signals from detectors and their further processing was possible due

to the application of Unical GPC Software, Version 4.06 (Viscotek). The checking of the correctness of chromatographic system performance as well as the calibration and analytical procedures were carried out in accordance with [18].

RESULTS AND DISCUSSION

As it was mentioned above, for GPC examination DMAC/LiCl solvent system has been applied as an eluent, in accordance with the references [16, 19] and our previous studies [14].

Considering the fact that in GPC analysis of polymers with very high molecular weights, BC included [20, 21], chromatographic parameters such as flow rate, injection volume and concentration of the solution significantly affect the measurement correctness, their optimisation has been carried out. It is known that both too high flow rate and concentration can deform the results obtained, among others due to the phenomenon called "finger of viscosity" [22]. In the optimisation so called "broad standard" of $M_n = 517 \cdot 10^3$ g/mol and $M_w = 1877 \cdot 10^3$ g/mol has been applied, prepared from the mixture of "narrow" polystyrene (PS) standards. The following optimum parameters of GPC have been established: flow rate of eluent — 0.4 mL/min, concentration of the injected cellulose solution — 0.18 ± 0.01 mg/m, injection volume — 150 μL.

Following the previous studies [23—27] the polystyrene (PS) standards were applied for the calibration of GPC system. The results of analyses were calculated using universal calibration method [28]. In the studies, a set of eight PS standards showing Mp*) values from 7000 to 7 100 000 and polydispersity <1.1 (Polymer Laboratories Ltd. Shropshire, UK) was applied. The obtained Mark—Houwink equation for PS was $[\eta] = 19.6 \cdot 10^{-5} M^{0.638}$ which is in good accordance with the one obtained by Timpa $[\eta] = 17.3 \cdot 10^{-5} M^{0.642}$ [25]. It indicates that the GPC system has been properly prepared for BC analyses.

Two series of microbiological cultures have been prepared. In the first one, BC samples were obtained at different temperatures of the process, *i.e.* 20 °C, 25 °C, 30 °C and 35 °C with a standard composition of the culture medium, pH = 6.0 and 7 days' duration.

In the second series pH of the culture medium was 3.8, 4.8, 6.0 and 7.5. It was regulated by changing the amounts of citric acid and disodium phosphate used in the process. The components were the same as in the standard culture medium. The experiments were carried out at 30 °C for 7 days. For each sample its molecular characteristic, intrinsic viscosity [η] and α-cellulose content as well as the productivity of the process were determined.

T a b l e 1. Effect of biosynthesis temperature on molecular characteristic of BC (DP_n, DP_w and R_{gz} values are mean of triple ones)

Biosynthesis temperature	DP_n	DP_w	DP_w/DP_n	R_{gz}, nm
20 °C	2600±60	7700±70	2.9	91±1
25 °C	2700±50	8640±80	3.2	93±2
30 °C	2930±30	9820±60	3.3	98±2
35 °C	2100±50	6810±100	3.3	84±2

The results obtained for the samples synthesized at different temperatures have been presented in Table 1 and also as a histogram (Fig. 1) for the better illustration of the changes occurring. These data prove that both productivity of the process and parameters of the molecular characteristics have been affected by the temperature of the process. As the temperature increases from 20 °C to 30 °C, the productivity of the process also grows from 1.64 to 3.20 g/L. A further increase of the temperature up to 35 °C causes a drastic lowering of productivity to 1.88 g/L. On the other hand, α-cellulose content remains independent on temperature and is high for all the samples, exceeding the value 99.5%. Molecular parameters also reach the highest values at 30 °C (DP_n = 2900, DP_w = 9820). A similar tendency is observed for [η] and R_{gz} values, being at the temperature 30 °C 12.2 dl/g and 98 nm respectively.

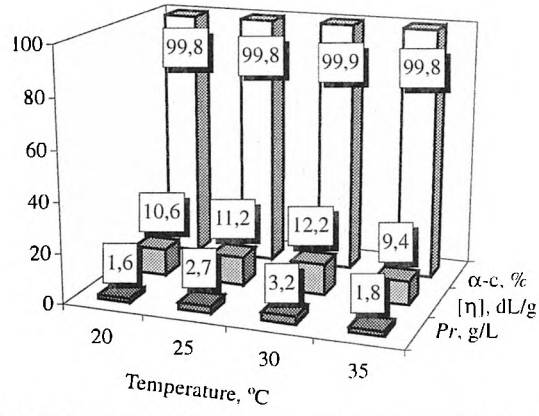

Fig. 1. Effect of biosynthesis temperature on cellulose productivity (Pr), α-cellulose content (α-c) and intrinsic viscosity ([η])

Changes of *MWD* are illustrated in Fig. 2. All the curves obtained at different temperatures are unimodal and have similar shapes. A slight differentiation can be noticed only with regard to the location of the curves. *MWD* curve 3 at 30 °C corresponds to the highest molecular weight values.

*) M_p — molecular weight of most numerous fraction of macromolecules in a given sample, so most, probable M_w value in the set examined. This value is used by the producers of PS standards

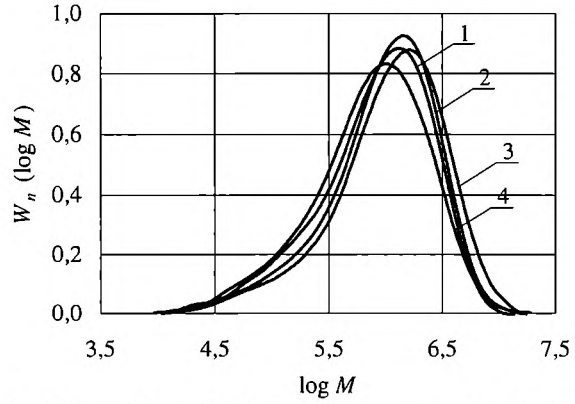

Fig. 2. Effect of biosynthesis temperature on molecular weight distribution of bacterial cellulose; temperature 1 — 20 °C, 2 — 25 °C, 3 — 30 °C, 4 — 35 °C (W_n — share of the given M value)

Table 2. Effect of medium pH on molecular characteristic of BC (DP_n, DP_w and R_{gz} values are mean of triple ones)

Medium pH	DP_n	DP_w	DP_w/DP_n	R_{gz}, nm
3.8	3120±100	9350±110	3.0	97±1
4.8	2960±90	9540±100	3.2	98±2
6.0	2930±50	9820±90	3.3	99±2
7.5	2600±70	9290±100	3.5	96±2

Table 2 shows the effect of the culture medium on the molecular characteristics of BC for different pH values of the culture medium. The results of the process productivity, α-cellulose content and [η] have been presented in the form of a histogram (Fig. 3). Molecular characteristics parameters are affected only slightly by pH reaching the lowest values for pH = 7.5 (DP_n = 2600, DP_w = 9200, R_{gz} = 96 nm). A similarly slight effect of pH on [η] of BC can be observed. The lowest [η] value (11.9 dl/g) was noted for pH = 7.5, whereas in other cases [η] value remained constant (12.2 dl/g); α-cellulose content for all BC samples was very high, exceeding 99.5%.

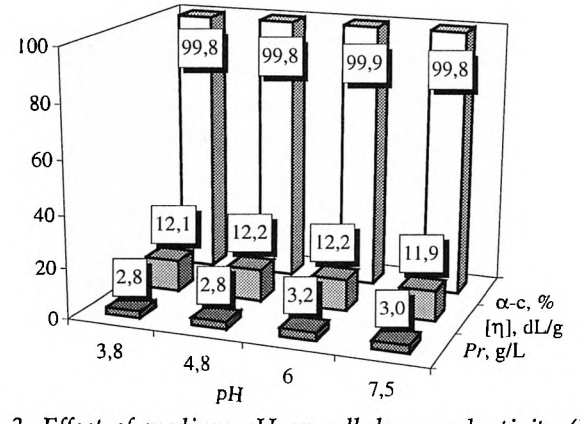

Fig. 3. Effect of medium pH on cellulose productivity (Pr), α-cellulose content (α-c) and intrinsic viscosity ([η])

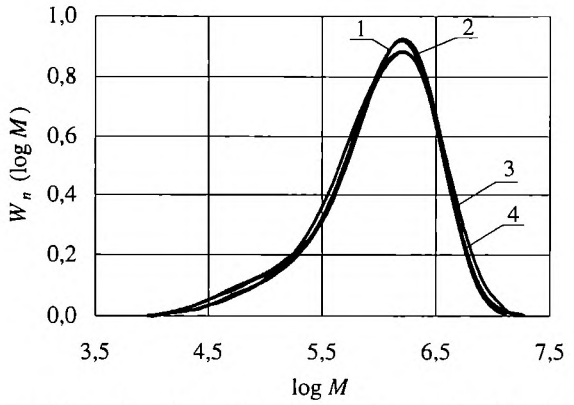

Fig. 4. Effect of medium pH on molecular weight distribution of bacterial cellulose; pH: 1 — 3.8; 2 — 4.8; 3 — 6.0; 5 — 7.5 (W_n — see Fig. 2)

MWD curves obtained for different pH values of the culture media have been presented in Fig. 4. Practically no effect of pH on the shapes and locations of *MWD* curves has been noticed.

CONCLUDING REMARKS

The characteristics of bacterial cellulose, undoubtedly superior in comparison with that of plant cellulose, makes this polymer an interesting object both of scientific investigation as well as of its practical application in a large scale. Taking into consideration the practical aspect, an optimisation of a biosynthesis process is necessary in order to improve the parameters characterising the process and the resulting product.

As we mentioned before, in our previous studies an optimum duration of the process and the most advantageous composition of the culture medium were determined [11].

The results of the present investigations prove that the temperature of biosynthesis significantly affects the physicochemical characteristics of the product. Thus, it has been found out that the optimum temperature of the process is 30 °C. The polymer obtained at this temperature is characterised by the highest polymerisation degree and the maximal value of [η]. As a result, any changes of the temperature of biosynthesis lead to the deterioration of the polymer characteristics. It should also be mentioned that the increase in the temperature by 5 °C is less advantageous than its lowering by 10 °C.

The examination of BC samples obtained at various pH values of the culture medium ranging from 3.8 to 7.5 showed that pH only slightly affects the properties of the product under investigation.

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