Bacterial cellulose produced by *Medusomyces gisevii* on glucose and sucrose: biosynthesis and structural properties

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Abstract In this work, the effects of carbon sources and culture media on the production and structural properties of bacterial cellulose (BC) synthesized by *Medusomyces gisevii* have been studied. The culture medium was composed of different initial concentrations of glucose or sucrose dissolved in 0.4% extract of plain green tea. Parameters of the culture media (titratable acidity, substrate conversion degree etc.) were monitored daily for 20 days of cultivation. The BC pellicles produced on different carbon sources were characterized in terms of biomass yield, crystallinity and morphology by field emission scanning electron microscopy (FE-SEM), atomic force microscopy and X-ray diffraction. Our results showed that *Medusomyces gisevii* had higher BC yields in media with sugar concentrations close to 10 g L^{-1} after a 18–20 days incubation period. Glucose in general lead to a higher BC yield (173 g L^{-1}) compared to sucrose (163.5 g L^{-1}). The BC crystallinity degree and surface roughness were higher in the samples synthetized from sucrose. Obtained FE-SEM micrographs show that the BC pellicles synthesized in the sucrose media contained densely packed tangles of cellulose fibrils whereas the BC produced in the glucose media displayed rather linear geometry of the BC fibrils without noticeable aggregates.

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Introduction

Bacterial cellulose (BC), made up of β-1, 4 linked glucose units, is a very versatile natural material perfectly suitable for numerous technological and medical applications (Gupte et al. 2021; Basu et al. 2019). Among notable features of BC are high crystallinity and high purity, impressive water-holding capacity and low production costs, as well as outstanding mechanical properties (Wang et al. 2018; Mikkelsen et al. 2009).

It is known that bacterial cellulose synthases are composed of several functionally distinct subunits

regulated by multiple factors, including cyclic diguanylic acid, diguanylate cyclases and phosphodiesterases maintaining its intracellular turnover (Nakai et al. 1999). Research on the genetics and biochemistry of BC formation conducted in the past two decades brought significant contributions to the elucidation of the mechanisms of BC biogenesis (Mikkelsen et al. 2009).

Among carbon sources, glucose is considered as the principal one for BC production. However, in many cases sucrose and other sugars may be biotechnologically and economically preferable substrates for BC biosynthesis (Hodel et al. 2020). Opportunities for the enhancement of BC production by using alternative substrates have been investigated by many research groups. Singhsa et al. demonstrated suitability of many mono- and disaccharides for BC production by *Komagataeibacter xylinus* (Singhsa et al. 2018). Nakai et al. confirmed that the carbon source used for BC production can significantly impact the process variables and outcomes (Nakai et al. 1999). Basu et al. recently argued that BC yields on sucrose may reach ~ 40 gL^{-1}, which is much higher than the typically reported values of ~ 10 gL^{-1} (Basu et al. 2019).

Despite the existing research, peculiarities of BC synthesis using carbon sources other than glucose are still poorly investigated. Among BC producers (*Acetobacter, Gluconacetobacter aka Komagataeibacter, Rhizobium, Agrobacterium, Sarcina,* etc.) there are significant variations in production abilities and in the synthetized BC properties. The fact that different carbon sources have different molecular weights, chemical structures and bioavailability, leads to even higher variations in biosynthetic rates and undesired fluctuations in BC structural characteristics (Singhsa et al. 2018; Wang et al. 2018; Erbas Kiziltas et al. 2015). All these factors strongly restrict the commercial efficacy of BC production. Optimization of culture medium composition, pH, carbon course, and ionic strength thus remain actual tasks since many BC production technologies are critically hindered by the slow production rate and a significant batch-to-batch variability of the yield (Li et al. 2021; Semjonovs et al. 2017).

Obviously, it would be advantageous to gain a better understanding and greater control over the physicochemical characteristics of the cellulose produced by microorganisms (Abdelraof et al. 2019). This stipulates careful examination of the BC-producer's ability to metabolize different carbon sources and their precise impact on the synthesized cellulose qualities.

In some our previous studies, we reported about the molecular assembly, architecture and mechanical behavior of native and modified BC synthetized by *Medusomyces gisevii* (Pogorelova et al. 2020). This symbiotic consortium, also known as "tea fungus" or "Kombucha fungus", predominantly consists of acetic acid bacteria (*Acetobacter, Komagataeibacter*) and yeasts *Zygosaccharomyces* (Marsh et al. 2014). Yeast cells, consuming carbohydrates, form ethyl alcohol and vitamins, thus supporting and facilitating the metabolic activity of the acetic acid bacteria. The latter, in turn, perform partial oxidation of carbohydrates and alcohol, leading to the accumulation of acetate, gluconate and citrate. These and other bacteria-derived organic acids serve as an additional carbon source for the yeast cells. Due to these mutual regulation processes, the symbiotic populations of Kombucha fungus co-exist as a self-supporting dynamic system.

Mostly, high-quality BC can be synthesized under static cultivation conditions. In this work, we attempted a systematic characterization and modification of the static BC production conditions by *Medusomyces gisevii* in order to understand the interplay of different parameters more clearly. In particular, we compared the impact of different concentrations of glucose and sucrose on the BC yield and production rate, on the substrate conversion degree, on the evolution of pH- and titratable acidity, as well as on the crystallinity degree, microfibrillar organization and other structural features of the synthesized BC. Besides theoretical interest, the collected data should benefit the existing BC production technologies and could contribute to development of advantageous approaches in the future.

Materials and methods

Culture medium composition and biosynthetic process

Symbiotic microbial consortium *Medusomyces gisevii*, also known as "Kombucha fungus" was acquired from the Scientific Center Kurchatov

Institute–Research Institute for Genetics and Selection of Industrial Microorganisms (Russia). This steady microbial community is composed of 15–30 genera represented mainly by yeasts (predominantly *Zygosaccharomyces* sp.) and the acetic acid bacteria (mainly *Gluconacetobacter* sp. and *Acetobacter* sp.). The exact species composition of the culture is detailed elsewhere (Jarrell et al. 2000; Jayabalan et al. 2014).

The used culture medium consisted of glucose or sucrose (Sigma-Aldrich Co.) at various w/w concentrations (5.0%, 7.5%, 10.0%, 15.0%, and 17.5%) dissolved in 0.4% extract of plain green tea (Woodbury Sugar Shed Co. USA). The tea solution was prepared by extracting 4 g dry green tea with 1L of hot (80 °C) tap water for 15 min, resulting in approx. 1.21 g•L^{-1} green-tea extractives in the medium. This medium is "classical" for *Medusomyces* since it does not require further sterilization, so the cultivation can be done under non-sterile conditions.

The BC biosynthesis was carried out by *Medusomyces* under batch cultivation conditions within cuboid polypropylene tanks (15 cm height × 17 cm width × 10 cm length) at 25 ± 2 °C. The medium was inoculated with a suspension of *Medusomyces* biomass previously adapted (seven days long) for the corresponding carbon source. The initial volume of the culture medium in the tank was 1.5L, and the initial ratio of the inoculum and the culture medium was adjusted to 1:3 (v/v). The active (titratable) acidity was daily followed but never adjusted during cultivation.

Synthetized BC was formed as a swollen gel pellicle (film) at the air–water interface, which thickness increased with longer cultivation durations. BC pellicles were collected and examined daily starting from 4th day to until 20th day of cultivation. After desired cultivation period, the sample to be examined was vigorously stirred for 10–15 min to remove the attached bacterial cells. BC pellicles at the air/liquid boundary were separated from the culture liquid, gently squeezed out, and washed with deionized water until neutral pH values (close to 7.0) were reached. Each gel sample was collected and analyzed in triplicate (i.e., from three different flasks).

The rinsed material was incubated in the same volume of 0.1 M NaOH at 80 °C for 90 min to remove the remaining microbial cells. This step was repeated four times until a colorless transparent BC pellicle was obtained (Fig. 1a–b). The resulting BC pellicle was rinsed with deionized water until neutral pH values (close to 7.0). The weight of the wet cellulose gel pellicles was measured using a laboratory balance. The washed BC was pre-frozen in a refrigerator at −70 °C for 6 h and then freeze-dried at −75 °C for 48 h. Hermetically closed samples of lyophilized cellulose (Fig. 1c–d) were stored in plastic containers until further tests.

Fig. 1 The appearance of the BC gel film samples synthesized on glucose (**a**, **c**) and sucrose (**b**, **d**) before and after lyophilization. The two upper pictures show the wet purified BC pellicles, and the pictures below represent the samples dehydrated by freeze-drying
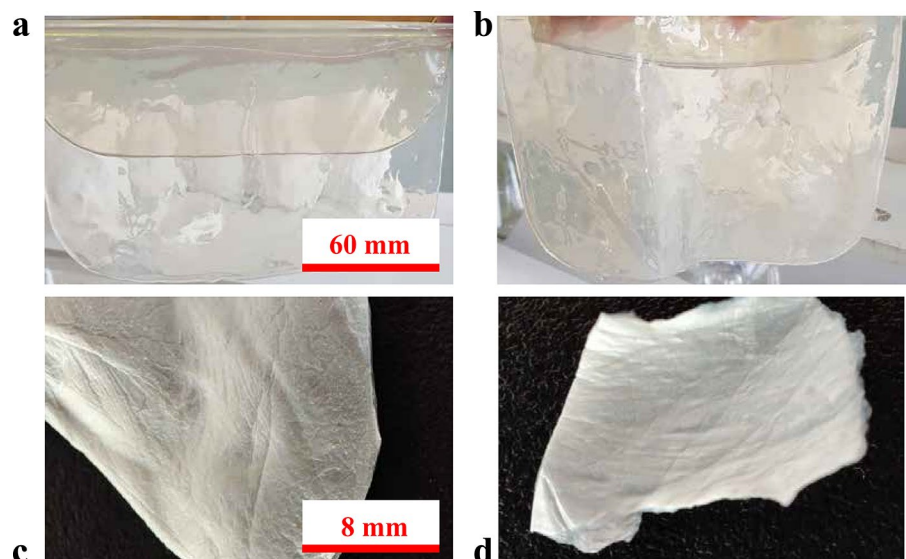
Determination of the carbon source concentration, substrate conversion and BC yield

Glucose concentration in the culture medium was determined by the glucose oxidase method using the commercial kit Novokarb (Vector-Best, Russia). Glucose oxidase catalyzes the transfer of two hydrogen atoms from the first carbon atom of glucose to the oxygen dissolved in the liquid reagent. In the course of the reaction, hydrogen peroxide is formed in equimolar amounts. Thus, the concentration of formed hydrogen peroxide is exactly equal to the determined concentration of glucose. After adding the sample to the working solution at a temperature of 37 ± 2 °C, a colored compound was formed within 20 min. The optical density of the final product at a light wavelength of 510 nm was determined with a photoelectric photometer KFK-3-01-"ZOMZ" and expressed in concentration units using a calibration curve.

Determination of sucrose concentration in the culture medium was performed by measuring the difference in reducing substances before and after optical inversion. The content of reducing substances was determined by the thiosulfate reaction, by the method of back titration, as described by Peris (2016). This method is currently regarded as standard one throughout the Commonwealth of Independent States (GOST 29248-91Ю: Canned milk. Iodometric method for the determination of sugars).

More specifically, the mass fraction of sucrose (C) as a percentage was calculated using the formula:

$$C = \frac{\frac{(V_1 - V_2)}{2} \times 0.99 \times 0.0171 \times 100}{m} \qquad (1)$$

where V1 is the amount of 0.1N sodium thiosulfate solution used for titration of the control sample, ml; V2 is the amount of 1N sodium thiosulfate solution used for titration of the test sample, ml; 0.0171—titer of sodium thiosulfate solution, expressed in grams of sucrose; 100 is the coefficient of recalculation in percent; 0.99 is a correction coefficient for the side reaction of fructose with iodine; m is the mass of the sample in grams (corresponding to the filtrate taken for titration).

The BC yield was calculated as wet BC weight per liter of the culture medium. The carbon conversion rates were defined as the weight of freeze-dried BC divided by the dry weight of the added carbon sources in the original medium.

Each experimental variant was measured in triplicate (samples were always collected from different flasks). The calculated mean values were processed and visualized by using the software packages Excel2010 (Microsoft Corp.) and STATISTICA 6.0 (StatSoft GmbH, Germany).

Determination of pH and titratable acidity of the media

The pH measurements were conducted using a laboratory pH meter (Mettler Toledo™ FiveEasy™). The initial level of acidity was determined directly after inoculation of the nutrient media. The titratable acidity was determined and expressed as Thörner degrees (°T). The Thörner degree is defined as the volume (in mL) of 0.1 M NaOH required to induce a color change (to pale pink) of the pH indicator (1% phenolphthalein in 70% aqueous ethanol) mixed with 100 mL of the tested sample (Navrátil et al. 2004; Tamime and Robinson 1985).

Characterization of bacterial cellulose

The samples of freeze-dried cellulose gel film synthesized on glucose or sucrose for 8 days were used to study and to compare the micro-morphological features of BC. First, the specimens were cut into 2×2 mm pieces by using a sharp blade. Then, the samples were mounted and examined using a scanning electron microscope JCM 5700 (JEOL Ltd. Japan) equipped with JED-2300 Analysis Station Plus, which can perform elemental analysis by detecting characteristic X-rays derived from the specimen. In this case the primary electron beam of the imaging system interacts with the sample and generates low-energy secondary electrons with characteristics that reflect the topographic nature of the specimen. The backscattered primary electrons produce images with a high degree of atomic number (Z) contrast. Ionized atoms can relax by electron shell-to-shell transitions, which lead to either X-ray emission or Auger electron ejection. The emitted X-rays were measured by the EDX detector to provide elemental information from the top few µm of the sample.

For the reduction of sample destruction by the primary electron beam upon observation, in our study the measurements were conducted at 5 kV accelerating voltage and using working distance of 10 mm. All images were taken in the plane perpendicular to the sample surface. Images were randomly selected from at least three different positions of three individual samples, using magnifications ranging from ×500 to ×20,000.

Additional information about sample surface topology and fibrillar structure was obtained using an atomic force (AFM) microscope NTEGRA Prima (NT-MDT Spectrum Instruments, Russia) in a semi-contact mode. The core of the technique is the registration of the interatomic forces acting between the studied surface and the probe sensor (cantilever) fluctuating at a resonant frequency. Mechanical oscillations of the cantilever were excited by means of the piezoceramic drive. Amplitude and a phase of these fluctuations were detected using an optical sensor that registers the deviation of the cantilever's reflected laser beam. The scanning was performed in the air by HA-NC Etalon Series cantilever with a characteristic hardness of 3.5 N/m.

Together with the "height" signal, allowing the investigation of the sample topography in three space coordinates, the "mag" signal was registered for better visualization of fine surface irregularities. Furthermore, the "mag" mode allows the detection of minor surface inhomogeneities, as it simulates the constant height mode, revealing the deviation of the probe when moving between adjacent surface points. The obtained signal was measured as a change in the photocurrent (nA) between its upper and lower section of optical sensor. Image processing was performed using the Gwyddion software developed by David Nečas and Petr Klapetek and distributed under the GNU GPL license (Nečas and Klapetek 2012).

To assess the supramolecular structure of bacterial cellulose, X-ray diffraction (XRD) measurements of freeze-dried BC samples previously grown in nutrient media of glucose and sucrose were performed on a Shimadzu XRD-7000 diffractometer with monochromated $Cu_{K\alpha}$ radiation in the θ–2θ mode. The diffractogram was recorded between 2θ angles of 10° to 30°. The applied tube voltage was 40 kV, current 40 mA. Deflection, scattering and receiving slits were set to 1°, 1°, and 0.15 mm, respectively.

Using the Wulf-Bragg formula, for each diffraction angle, the parameter d, the distance between atomic planes, was calculated (Fang and Catchmark 2014).

The crystallite size (Cs) was calculated using the Scherrer formula

$$C_s = \frac{0.94 \times \lambda}{D_{fwhm} \times cos\theta} \tag{2}$$

where the coefficient of proportionality 0.94 is chosen based on the assumption of a spherical shape of crystallites, θ is the diffraction angle scattering (Sumadiyasa and Manuaba 2018).

Data processing and pseudo-Voigt fitting of the XRD peaks was performed in Fityk software under the assumption that the reflection of the (100), (010) and (110) crystal planes of the cellulose corresponds to 14.50°, 16.70° and 22.50 of 2θ respectively (Wojdyr 2010). Therefore, the amorphous area was considered to be centered at approximately 18.5°.

The used pseudo-Voigt function pV(x) can be described as:

$$pV(x) = \eta L(x) + (1 - \eta)G(x) \tag{3}$$

where the weighting parameter η determines the ratio of the Lorentzian (L) and Gaussian (G) fitting components (David 1986; Balzar and Ledbetter 1993).

The obtained integral intensities were used to calculate the degree of crystallinity by the Ruland-Vonk method (Bordun et al. 2021). This method makes it possible to obtain the degree of crystallinity (SC) from the ratio of the area of the crystalline phase to the total area under the diffraction curve, which includes scattering by the amorphous and crystalline phases. The initial stage of the calculation consisted in determining the position of the maximum and fitting the peaks of the crystalline component. Next, the numerical data on the areas of the crystalline and amorphous phases, the width of the reflections at half maximum, and the diffraction angles were refined and analyzed using the Fityk program. The background correction was performed by subtracting the baseline values connecting the horizontal sections in the lower part of the spectrum.

Results and discussion

BC yield using different carbon sources

The BC-synthetic activity of *Medusomyces gisevii* was evaluated for two different carbon sources (glucose and sucrose) at different initial sugar concentrations ranging from 5.0 to 17.5%. The amount (wet mass) of biosynthesized BC and the rate of its biosynthesis were determined every 24 h at twelve different time points during cultivation. The results are presented in Fig. 2.

Figure 2a shows the BC yields that were daily monitored upon *Medusomyces gisevii* cultivation on different concentrations of glucose and sucrose. Distinct BC pellicles formed in the air–water interface starting from the 4th day of cultivation. For both used carbon sources, the production of BC increased with an increasing initial concentration of

the nutrient medium up to 10% but then decreased at higher concentrations.

The largest yields of BC (173 gL^{-1}) were obtained on glucose after the 18th day of cultivation at the starting concentrations close to 8.0%. Lower BC production was observed at both lower and higher initial glucose concentrations. The obtained concentration optimum values are higher than those previously reported by other groups (~5.0%), which may be explained by differences in the microorganisms used (Younesi et al. 2019). A possible reason for the decreased production of BC at glucose levels of less than 5% is that glucose was the only available energy source in the medium for some microorganisms comprising *Medusomyces gisevii*. The inhibition of the BC production at initial glucose concentrations of more than 14.0% can be attributed to high osmotic pressure and lower water activity.

On sucrose, the maximal yield of the synthesized BC pellicle of 163.5 gL^{-1} was reached on the 20th

Fig. 2 a Wet mass dynamics of the BC produced during 20 days of cultivation using different initial concentrations of glucose and sucrose (displayed on the axis) in the culture medium. **b**: The rate of BC-biosynthesis (calculated as dry mass produced per liter of culture medium per day) on media containing different initial concentrations of glucose or sucrose
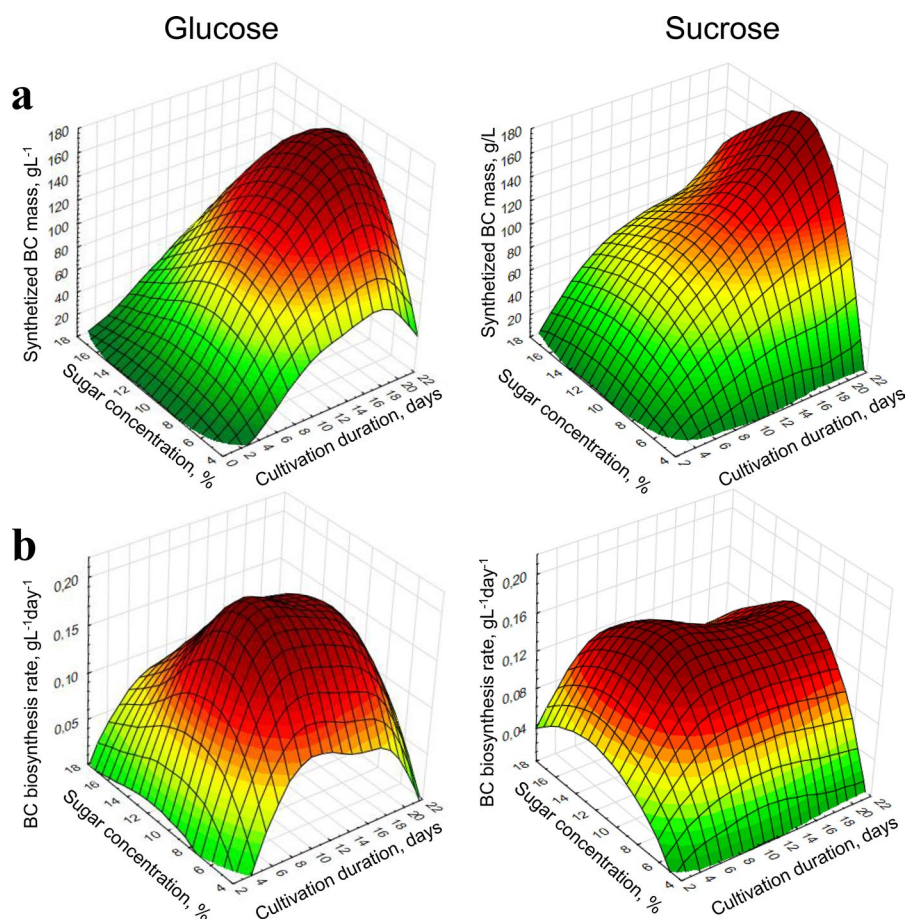
day, when initial sucrose concentrations were close to 10.0%. Lower initial sucrose concentrations correlated with decreased cellulose biosynthesis. For instance, the BC yield did not exceed 27.0 gL^{-1} for 5.0% sucrose after the 20th day of cultivation. Similarly, at high initial sucrose concentrations (15.0% and 17.5%), decreased BC yield values were measured during the entire process (20 days), which may be associated with the inhibition of the symbiotic acetic acid bacteria. This conclusion is also supported by a lower rate of the culture medium acidification (as will be discussed in the sections below).

The measured differences in the BC yields between the glucose and sucrose variants are presumably related to the differences in BC biosynthetic pathways and the level of involvement of glucokinase, phosphoglucomutase and uridine triphosphate (UTP)-glucose-1-phosphate uridylyltransferase. It is known that glucose transformation to glucose-6-phosphate, glucose-1-phosphate, uridine diphosphate (UDP)-glucose occurs prior to incorporation to unbranched beta-1,4-D-glucan chains (i.e., BC) with the aid of cellulose synthases (Wang et al. 2018). The sucrose transformation pathways in *Medusomyces* are not well studied yet.

Our results may indicate that sucrose exhibits lower efficiency as a substrate for BC biosynthesis compared to glucose. In particular, insufficient concentration of sucrose may reduce the intensity of alcoholic fermentation by symbiotic *Saccharomyces* and *Zygosaccharomyces* species, which results in decrease in ethanol formation, thus diminishing ethanol's enhancing effects on BC biosynthesis (Cielecka et al. 2021; Li et al. 2012).

For all studied glucose concentrations, the highest yields were obtained upon BC pellicle removal on the 18th day of cultivation. Thereafter, a slight decrease in the mass of the BC pellicle was observed (well visible on the 20th day). This can be related to the partial destruction of the polymer matrix in the late culture. However, in the sucrose variants, no such BC degradation was observed after 18 days, probably due to slower carbon source utilization. A similar "postponed" sucrose contribution effect to the BC biosynthesis was reported by Mikkelsen et al. However, in their study, a single *Gluconacetobacter xylinus* strain produced BC on sucrose more readily (Mikkelsen et al. 2009).

BC biosynthesis rate depending on the carbon source

According to the results presented in Fig. 2, the highest BC biosynthesis rates were reached using 7.5–10.0% glucose nutrient media. A sharp increase in the BC production rate was observed on the 8th day for concentrations 7.5% and 10.0%, where the biosynthesis rate reached the maximum value of 0.23 gL^{-1} BC dry mass per day, which was also consistent with the wet mass BC dynamics (Fig. 2a). For comparison, the BC production rate in 15.0–17.5% glucose on the 8th day reached 0.098 gL^{-1} day^{-1}. After 8th day, the biosynthesis rate decreased for all studied glucose concentrations, so that on the 20th day it was 1.5–1.9 times lower compared to the peak values.

For sucrose, the maximal BC biosynthesis rate was determined on the 8th day of the cultivation, reaching 0.165 gL^{-1} dry weight per day for 10.0% sucrose. On the 20th day the rate decreased by 10.9% (Fig. 2b). The highest BC yield and biosynthesis rate were typical for nutrient media containing 10.0% sucrose: the mass of BC pellicle—163.5 gL^{-1} and the biosynthesis rate—0.165 gL^{-1} dry weight per day on the 20th and 8th days, respectively. These results again imply a lower efficiency of sucrose as a substrate for BC biosynthesis compared to glucose. In some published studies, greater growth on sucrose was achieved, partially due to the consideration of molar mass. Moreover, when growing on sucrose, less substrate was "wasted" for the synthesis of organic acids (Yoshinaga et al. 1997; Nakai et al. 1999; Amor et al. 1995).

Sugar type effect on the substrate conversion degree

In the microbial cell, the carbon source from the culture medium is converted into UDP-glucose (a key precursor for the BC synthesis) through a series of enzymatic reactions. Unlike other sugars, D-glucose is not only a convenient energy source, but also as an ideal precursor for the cellulose biosynthesis (Masaoka et al. 1993). The rate of glucose consumption from the medium during the BC biosynthesis was shown to be fully consistent with the amount of BC produced (Aswini et al. 2020; Klemm et al. 2005; Gullo et al. 2019).

Dynamics of the substrate concentration in the culture media during the BC biosynthesis are shown in Fig. 3 for different initial concentrations of glucose and sucrose. The most significant decrease in the

Fig. 3 a: Dynamics of sugar concentrations during 20 days of cultivation in the culture media containing different initial concentrations of glucose and sucrose. **b**: The degree of the substrate conversion in the nutrient media containing different initial concentrations of glucose or sucrose
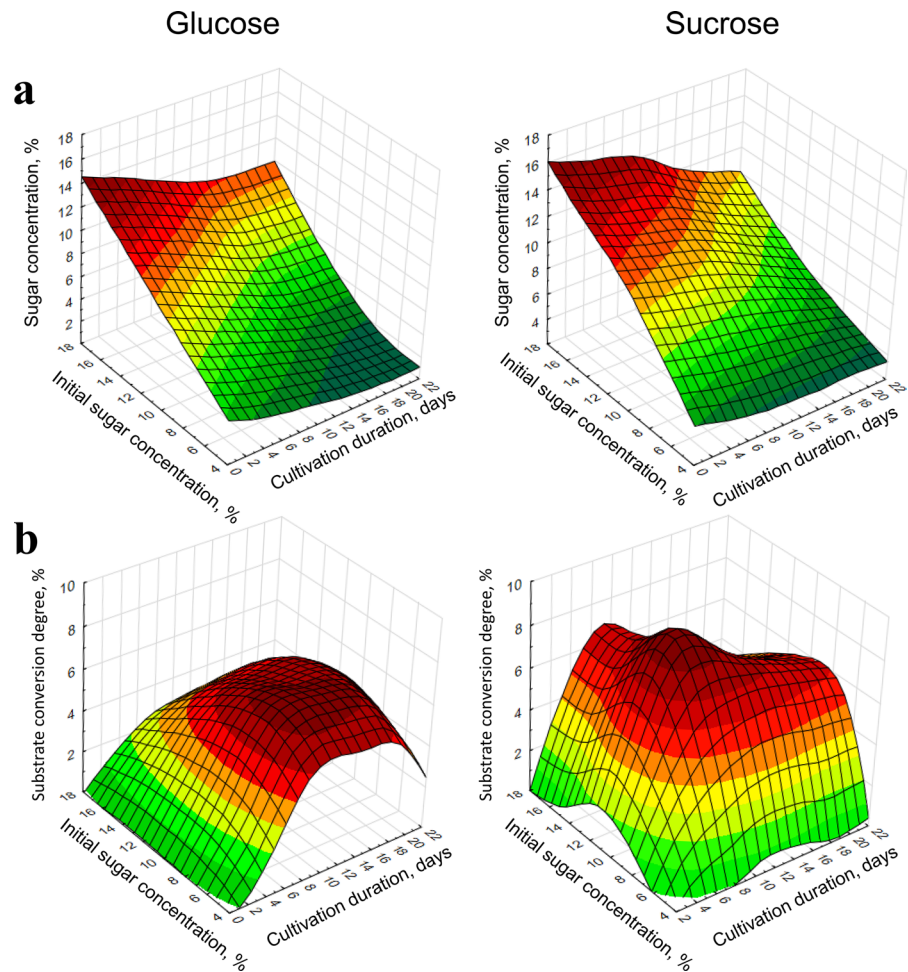
glucose content of 81.9% (from 64.0 to 11.6 gL^{-1}) was observed on the 20th day in the samples initially containing 7.5% glucose. Also, a substantial reduction in glucose content of 32.7% (from 141.2 to 95.4 gL^{-1}) was recorded for the highest initial glucose concentrations of 17.5%. Beginning from the 14th day of all tested nutrient media, the smallest glucose content changes in the culture media were determined, showing a decrease of less than 10 gL^{-1}. The minimal detected change in glucose throughout the 20-day period of cultivation occurred in the nutrient media containing initially the highest content of glucose (17.5%), which was consistent with the smallest mass of produced BC − 82.10 gL^{-1}.

The most comprehensive substrate utilization was determined when the *Medusomyces gisevii* was cultivated on the nutrient media containing 7.5% glucose and an initial concentration of 6.0%. Here, the

largest amount of BC was produced to the 18th day, reaching 172.65 gL^{-1}. Thus, the change in glucose in the culture media during biosynthesis was perfectly inversely correlated with the synthetized BC mass.

The substrate conversion degree (SCD) is one of the main indicators reflecting biosynthetic efficiency. In general, the maximal SCDs were found around the 8th day and then decreased during further cultivation. The highest SCD for 20 days of cultivation was calculated for nutrient media with 7.5% glucose and reached 9.04% (Fig. 3b).

At high glucose concentrations of 15.0 and 17.5%, the maximum SCD was reached much later, on the 18th day of cultivation, reaching 3.65% and 2.32%, respectively, which is significantly lower (> 2.5 times) than the measured absolute maximum on glucose. For all used glucose concentrations, the conversion degree decreased on the 20th day, which may be

associated with the previously mentioned onset of BC degradation after the 18th day of the experiment.

In the past, various carbon sources, including fructose, galactose, mannose, xylose, arabinose, sorbose, and others, were tested for their suitability for BC production by different microorganisms, but mostly with low conversion yields (Jonas and Farah 1998). However, one important disadvantage of utilizing glucose as a carbon source is related the pyrroloquinoline quinone cofactor-dependent glucose dehydrogenase (GDH) that converts glucose into gluconic acid (Shigematsu et al. 2005). The generation of gluconic acid consumes glucose in the nutrient medium and leads to undesired decrease of pH, thus additionally hampering BC biosynthesis (Verschuren et al. 2000).

On sucrose, the highest sugar consumption rates we measured around the 10th day of *Medusomyces* cultivation. In 10.0% sucrose the sucrose level decreased by 47.7% within 20 days of biosynthesis, which fits well to the reached highest total BC synthesis (163.5 gL^{-1}). The low activity of sucrase in the BC producers and the inability of the bacterial cell to transport sucrose across the cell membrane reported by (Zhong et al. 2013), means that sucrose in the medium must be first hydrolyzed to glucose and fructose. For this reason, we followed the glucose concentration changes also in the sucrose-based culture media. As expected, decrease in glucose concentration was observed during 10 days of cultivation. This trend may be due to the relatively slow rate of sucrose hydrolysis by yeast cells in comparison to glucose consumption speed, since glucose is used both as energy source and as a precursor of cellulose.

Starting from the 10th day of cultivation on a sucrose media, glucose accumulation in the culture media occurred. This effect was more pronounced in the variants with 15.0–17.5% sucrose, where on the 20th day, the glucose content reached 11.2 gL^{-1}. In the 7.5% and 10.0% sucrose variants the glucose concentration reached only 1.8 gL^{-1} at that time.

Furthermore, the efficiency of sucrose utilization was determined by calculating the degree of sucrose conversion at each stage of BC biosynthesis. The lowest degree of conversion was determined for the 5.0–17.5% sucrose-containing nutrient media during the entire biosynthesis process (20 days), which might be due to the non-optimal content of sucrose in the nutrient media for cellulose biosynthesis. Even at 1.11% glucose content on the 20th day of biosynthesis, the degree of substrate conversion for the 17.5% sucrose-containing nutrient media remained within 3.6%. This may indicate inhibition of synthetizing activity of the acetic acid bacteria when initial concentration of sucrose exceeded 14%. The maximum degree of sucrose conversion (11.29%) was determined for the 10% sucrose-containing nutrient media on the 8th day of biosynthesis. In the 7.5% sucrose samples the maximum degree of substrate conversion 8.16% was measured on the 10th day.

Dynamics of acidic products accumulation depending on the carbon source used

The formation of acidic by-products (especially acetic and gluconic acids) during the BC synthesis significantly impacts the substrate conversion into BC (Islam et al. 2017). In our experiments, the initial pH values of all media were relatively low (around 3.4) because pH was measured after medium inoculation with *Medusomyces* biomass. The samples having initial glucose concentrations between 5.0 and 17.5% demonstrated rapid pH drop from the very beginning and on the 20th day of cultivation reached pH values in the range 2.38 and 2.22. By contrast, in all tested sucrose media, the pH values remained relatively stable within first four days. Then, the level of later acidification of the media clearly correlated with the initial sucrose concentration (Fig. 4a). On the 20th day, the lowest pH value of sucrose media reached 2.76.

Since most by-products formed during BC biosynthesis are weak organic acids, the change in titratable acidity (TA) could be more informative for characterization of the substrate conversion and respectively BC biosynthesis. As expected, the measured TA values had in the in general the same trend as the pH data, and again exhibited "stability" period for the sucrose samples (Fig. 4b). The final values of titratable acidity were 323°T and 330°T for highest glucose concentrations 15.0% and 17.5% respectively, which was significantly higher compared to the corresponding sucrose variants (188°T and 215°T, respectively).

Thus, in contrast to the sucrose group, the glucose media displayed much faster pH changes and much rapid TA dynamics. The observed drop in pH values occurred relatively early in the whole studied concentration range with more acid metabolites formed, as evidenced by the comparison between pH and titratable acidity (Fig. 4).

Fig. 4 a: The dynamics of pH changes during 20 days of cultivation depending on initial concentration of glucose or sucrose in the culture medium. The time axis was reversed here for better visibility. **b**: The corresponding dynamics of titratable acidity measured in the studied samples
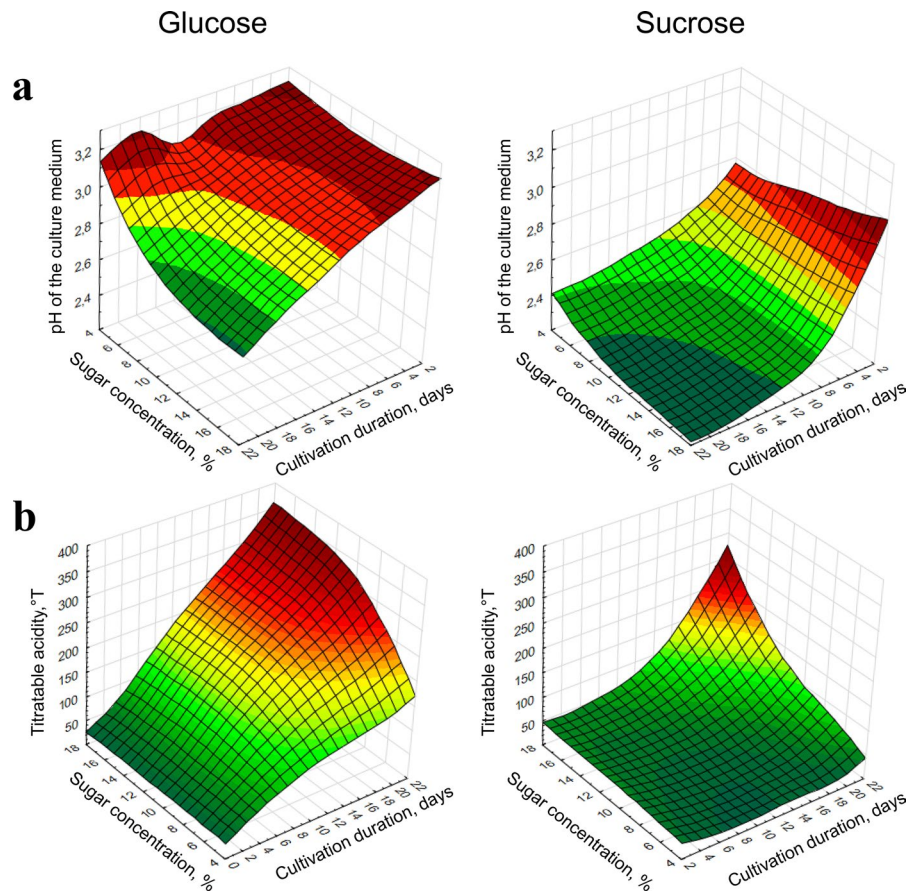
Characterization and comparison of BC synthetized on glucose and sucrose

Ribbon-like microfibrils characteristic for bacterial cellulose are known to be about 100 times thinner than those of plant cellulose. This fact, together with BC high crystallinity degree contributes to high porosity and an excellent water absorption capacity (more than 99% moisture content) of BC, allowing it to form a strong and flexible hydrogels (Hodel et al. 2020; Semjonovs et al. 2017).

Previous FE-SEM studies indicated that the BC fibrils are densely packed within the pellicles and the different used carbon sources had no significant effect on the micro-architecture of the resulting cellulose pellicles. Mikkelsen et al. demonstrated that the BC production rate was influenced by different carbon sources, but the formed BC specimens were indistinguishable in microscopic and molecular features (Mikkelsen et al. 2009).

Indeed, in our experiments, initial comparison of the microfibrils produced on glucose and sucrose media first did not reveal significant differences between them: all variants exhibited a randomly intertwined structure of relatively straight 10 µm long microfibrils. Primary surface profiling showed that both glucose- and sucrose-derived BC samples display a typical pattern of 120 nm microfibrils cross-wound in pairs (Fig. 5).

More detailed analysis showed though that the surface of samples synthesized on sucrose was more branched, dense and heterogeneous. It also displayed larger pores (up to 2 µm in diameter), and had overall higher roughness. The fibrils were found more entangled, more randomly intertwined and exhibited multiple bends. Surface of the samples grown on glucose was mainly composed by straight-shaped fibrils having a typical length of more than 50 µm (Fig. 6a).

Analysis of X-ray diffraction (XRD) data is helpful for better understanding of macro-characteristics and

Fig. 5 AFM image of bacterial cellulose synthesized on glucose and sucrose. **a**: two-dimensional models, **b**: surface profile created over the displayed line 1
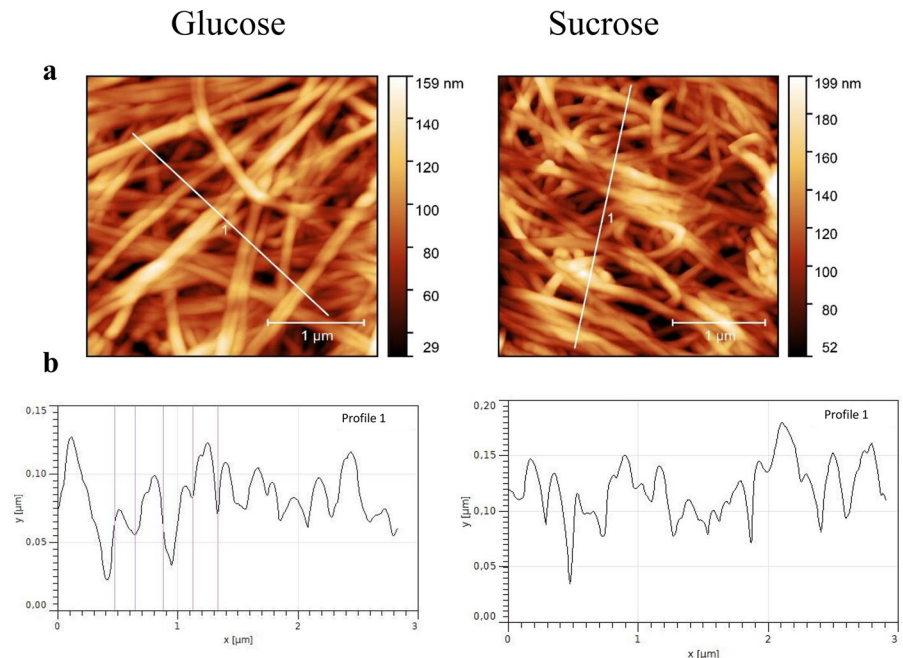
performance of the BC materials, such as strength, moisture absorption capacity, water holding capacity, and so on. Among the 4 polymorphic modifications of of cellulose, the Type I includes natural cellulose, which in turn is divided into Iα and Iβ subtypes. According to the Meyer-Misch model, Iβ cellulose has a monoclinic unit cell, while Iα cellulose is characterized by a single-fragment triclinic one. In celluloses from algae and bacteria, the low-symmetry Iα phase usually predominates, while the Iβ phase is major in celluloses from higher plants (wood, cotton, ramie, etc.).

From the literature, the crystal structure of bacterial cellulose is a combination of allomorphic modifications of crystal lattices, namely, triclinic, characteristic of cellulose Iα, and monoclinic, characteristic of cellulose Iβ (Fang and Catchmark 2014; Ruan et al. 2016; Sumadiyasa and Manuaba 2018; French 2014). Since native BC consists mainly of cellulose Iα (Ruan et al. 2016), for the above diffraction angles, the directions of crystal planes (100), (010), (110) corresponding to cellulose Iα were considered.

Similar to AFM and EF-SEM, the XRD indicated certain differences between the BC samples of different origin. In Fig. 6b the experimental points are shown to be approximated in the X-ray diffraction pattern built by the Fityk program. Thinner lines of

violet, black and green correspond to different components of the pseudo-Voigt model; the thick orange line represents the superposition all components.

Within the studied range the BC samples exhibited major reflection maxima at 14.50°, 16.70° and 22.50°. The collective contribution of the minor peaks corresponding to diffraction angles 14.2, 15.35, 16.65, 21.92, 23.5 and 23.6 was estimated to be 1–2% which was not substantial for the goals of this study.

Intensities for the (1 0 0) peak at 14.5° are stronger than those of the (1 1 0) peak at 22.5°. Further, the (0 1 0) peak, at 16.7° is substantially weaker than either of these peaks. This result somewhat differs from bacterial cellulose profiles reported previously (Younesi et al. 2019; Ruan et al. 2016). Similar deviation of bacterial cellulose patterns from the idealized ones was already mentioned (French 2014). However, our data are in a good accordance the patterns obtained by Fang and Catchmark in the reflection mode although for the transmission mode they reported a high intensity of both 010 and 110 peaks accompanied by a very low one for the 100 peak (Fang and Catchmark 2014).

The obtained integral intensities were used to calculate the degree of crystallinity by the Ruland-Wonk method. This method allows to obtain the degree of crystallinity (CI) from the ratio of the area of the
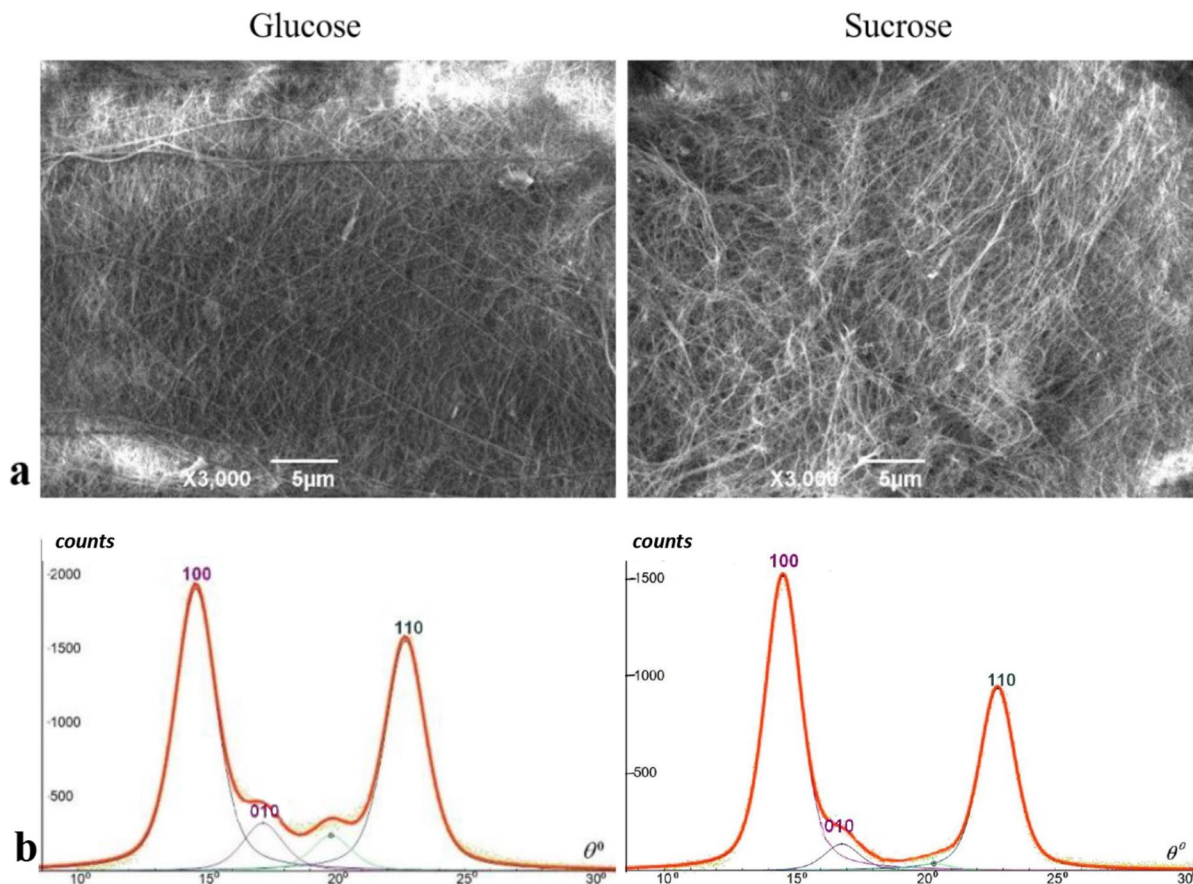
Glucose   Sucrose

Fig. 6 a FE-SEM image of BC synthesized on glucose and sucrose. **b** XRD patterns of BC synthesized on glucose and sucrose

crystalline phase to the total area under the diffraction curve, which includes scattering by the amorphous and crystalline phases. Here, we took into account that the integral intensity of each reflection in the X-ray diffraction pattern is proportional to the content of the crystalline phase, while the content of the amorphous component of the cellulose material is proportional to the diffuse scattering intensity.

The numerical values of the parameters of the degree of crystallinity (CI), interplanar spacing (d)

and crystallite size (Cs) for all samples are given in Table 1.

The reference diffraction pattern of amorphous cellulose received from the database ICDD PDF-4 (https://www.icdd.com/pdf-4/) is a symmetrical curve, about 25° wide, with the center of symmetry coming at a diffraction angle of 20°. Thus, to our knowledge, and also based on the ICDD PDF-4 records (e.g. 00-056-1719, 00-056-1718 and 00-060-1501), the intensity peaks at about 20° result mainly

Table 1 Parameters of the supramolecular structure of BC samples

	Glucose			Sucrose		
CI, %	94			98		
	(100)	(010)	(110)	(100)	(010)	(110)
d, nm	0.607	0.516	0.391	0.608	0.527	0.390
Cs, nm	4.56	4.62	4.79	5.29	5.35	5.56

due to the contribution from the amorphous cellulose phase which is here presumably much greater than the contributions from the Iα and Iβ phases. For this reason, we neglected this input in our crystallinity calculations. Comparison of the obtained diffraction data with the reference pattern implied that in all the tested samples the fraction of amorphous cellulose was insignificant. Consequently, all studied BC samples displayed a high degree of crystallinity (close to 100%).

It should be noted that for such highly crystalline materials, the width of the reflections is rather insignificant; their shape tends to be a vertical straight line. When calculating this parameter, the slope of the function graph and the width of the reflection at its half-height should be taken into account. Strictly speaking, the value of this parameter may also depend on the roughness of the sample surface. However, we believe that, when applied in the same controlled manner, this method is suitable for comparative analysis the studied samples.

Since the crystallographic texture, i.e. the orientation distribution of crystallographic planes in a sample can cause large systematic errors, XRD patterns from cellulose are never ideal. In spite of the fact that various mathematical forms have been proposed for correction and compensation for the deviations from ideality, the accurate measure of cellulose organization remains a difficult task (Sitepu 1991; Fancher et al. 2019; French 2020).

The size of cellulose crystallites depends on their origin, biosynthesis conditions and processing method. According to the literature data, larger cellulose crystals are formed with a minimum amount of accompanying (foreign) substances during the assembly of bacterial cellulose glucan chains. The calculated sizes of crystallites of bacterial cellulose synthesized on both sucrose and glucose are higher than those reported for the cotton cellulose (Fang and Catchmark 2014).

As we described above, the rate of BC formation on the sucrose substrate was lower than on the glucose substrate, which can presumably be a factor determining the degree of association of crystallites. This effect was visible in both Cs and CI values. In particular, crystallite sizes in the sucrose-related samples exceeded those in the glucose-derived ones by 16% (in average) in all directions. The degree of crystallinity also was found slightly higher for the

sucrose-derived BC, whereas the values of interplanar spacing (d) practically coincided between the studied groups.

In general, the results obtained from AFM, FE-SEM, and XRD were in mutual agreement that the BC samples synthetized on the sucrose-based media featured (a) thinning of microfibrils, (b) their higher entanglement, (c) a greater degree of disorder in their spatial orientation, (d) larger crystallite sizes and (e) slightly higher (~4.3%) crystallinity degree.

Conclusions

BC is growing in popularity owing to its applications in the food, medical, and cosmetics industry (Chernigova et al. 2019). After minor additional modifications, BC can be broadly applied as constituents of filter membranes, in wound dressings and in many other material compositions. However, its large-scale commercial use requires optimization of production technologies and reduction of production costs. Our study intended to shed more light on the influence of the carbon sources (glucose and sucrose) in the culture medium on the rate of BC production by *Medusomyces gisevii* as well as on the morphological/structural features of the obtained BC materials.

Analysis of the BC biosynthesis process showed that *Medusomyces gisevii* had higher BC yields in media with sugar concentrations close to 10 gL^{-1} after an incubation period for 18–20 days. Growth on glucose in general lead to a higher BC wet pellicle yield (173 gL^{-1}) compared to sucrose (163.5 gL^{-1}). On glucose, the maximum biosynthesis rate of $0.23 \text{ gL}^{-1} \text{ day}^{-1}$ (dry weight BC) and the highest substrate conversion degree of 9.04% were achieved on the 8th day. However, the maximum rate of BC biosynthesis on a nutrient medium containing 10.0% sucrose reached appreciable values of 0.165 g L^{-1} of dry mass per day, with the corresponding conversion degree equal to 11.3% on the 8th day. The formation of organic acid metabolites was significantly greater in glucose compared to sucrose. The BC crystallinity degree was higher in BC synthetized from sucrose.

In summary, we conclude that in spite of (in general) better BC production values reached on the glucose-based media, *Medusomyces gisevii* showed efficient cellulose synthesis and lower acid metabolites accumulation when utilized sucrose as the only

carbon source. The cellulose synthetized on sucrose was characterized by higher density, crystallinity and fibril entanglement. Moreover, it possessed larger pores (up to 2 μm in diameter), and had overall higher roughness, thus displaying some notable microstructural features that may be relevant for its practical applications.

Author contribution ID wrote and drafted the manuscript, including presentation and visualization of the results; NA performed literature survey, contributed to the interpretation of all data and to drafting of the manuscript; NP is the creator of the manuscript concept. She personally collected and analyzed all microbiological data and performed atomic force microscopy; ER performed FE-SEM, AFM and XRD. He analyzed and interpreted the obtained diffraction data. All authors approved the final version of the manuscript.

Declarations

Conflict of interest The authors declare no conflicts of interests with respect to this manuscript.

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