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Effect of *Gluconacetobacter xylinus* cultivation conditions on the selected properties of bacterial cellulose

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The aim of the study was to analyze the changes in the parameters of bacterial cultures and bacterial cellulose (BC) synthesized by four reference strains of *Gluconacetobacter xylinus* during 31-day cultivation in stationary conditions. The study showed that the most visible changes in the analyzed parameters of BC, regardless of the bacterial strain used for their synthesis, were observed in the first 10–14 days of the experiment. It was also revealed, that among parameters showing dependence associated with the particular bacterial strain were the rate and period of BC synthesis, the growth rate of bacteria anchored to the cellulose fibrils, the capacity to absorb water and the water release rate. The results presented in this work may be useful in the selection of optimum culturing conditions and period from the point of view of good efficiency of the cellulose synthesis process.

Keywords: bacterial cellulose, bacterial culture, cultivation time, culture conditions, *Gluconacetobacter xylinus*.

INTRODUCTION

Gluconacetobacter xylinus is one of the most commonly studied microorganisms with the ability to synthesize bacterial cellulose (BC)[1]. It is a Gram-negative bacterium, strictly aerobic, capable of producing cellulose extracellularly at temperatures between 25 and 30°C and pH from 3 to 7, using different sources of carbon and nitrogen[2]. This bacterium was also reported as the most efficient BC producer[3]. In terms of chemical structure, bacterial cellulose is identical to that produced by plants. However, it exhibits a higher crystallinity[4], water-holding capacity[5], degree of polymerization[6], mechanical strength and purity[2]. When the bacteria are cultivated in static culture conditions, the BC is synthetized as a cellulose pellicle situated on the broth surface. In this case it is assumed that the bacteria immobilize themselves in the cellulose to maintain their location between a substrate-rich surface and the oxygen-rich air[7].

BC is applied in such areas as biomedicine, paper production, textile industry and environmental protection[8, 9]. However, the relatively high cost of BC production limits its application in the production of high value-added products and specialty chemicals[7, 10, 11]. The yield of a BC biosynthesis process depends on such factors as temperature, ratio of the surface area to the volume of the substrate and on the time of synthesis[12, 13]. It is considered that a reduction of the cost of BC synthesis can be achieved mainly by improving fermentation efficiency[14]. For this purpose, various studies involving modification and optimization of the culture conditions e.g. medium composition, temperature of incubation or incubation time and the development of novel technologies have been conducted intensively all over the word[3, 7, 12, 15]. Based on the literature review, it was found that so far there have been few data describing the most important changes occurring during the cultivation of *G. xylinus* and the biosynthesis of cellulose. Some of the information is offered in the study by Sheykhnazaria et al.[16] (FT-IR spectra, X-ray diffraction patterns, microorganism morphology and its distribution in the cellulose after 7, 14 and 21 days of the culture) and by Hornung et al.[7] (dry mass of the BC layer, concentration of glucose in the substrate solution or number of cells immobilized in the cellulose, measured 30–50 days of the culture, depending on the experiment). However, to the best of our knowledge, no analysis showing the dependency between BC parameters and the *G. xylinus* strain has so far been published.

In order to optimize the production of BC, it is necessary to characterize microbial growth, product formation and substrate utilization by the microorganisms. In addition, it is imperative to identify how these processes are affected by the time of bacterial cultivation[7]. The determination of a such dependence would be beneficial in establishing the basic conditions for *G. xylinus* culture in order to select the most appropriate culturing period in terms of good efficiency of the BC synthesis process and to obtain cellulose with the desired properties. Therefore, the aim of the study was to analyze the changes in the basic parameters of cultures and the cellulose synthesized by various strains of *G. xylinus* during 31-day cultivation in stationary conditions. The investigation whether the changes in physicochemical properties differ between individual strains, and if so, whether this variation is associated with specific parameters relating to BC biosynthesis, was also included.

MATERIAL AND METHODS

Microorganisms and culture conditions

Prior to the experiment, 7-day cultures of *G. xylinus* ATCC 53524 (American Type Culture Collection), DSM 5602 (Deutsche Sammlung von Mikroorganismen und Zellkulturen – German Collection of Microorganisms and Cell Cultures), *G. xylinus* DSM 46602 and *G. xylinus* DSM 46604 were shaken and 100 μL of the obtained bacterial suspensions were used to inoculate 25 mL of Herstin-Schramm (HS) medium in 50 mL plastic tubes

with caps featuring 8 holes and a specific capillary pore filter membrane with a pore size of 0.2 μm providing gas exchange (CELLSTAR®CELLreactor, Polypropylene Filter Top Tube, Greiner Bio-One, USA). The BC synthesis was conducted at 28°C for 31 days. The BC pellicles were harvested from the medium after 3 days of cultivation and then after each 2 days, rinsed with water, wiped carefully with filter paper and weighed on an analytical balance (WTB 2000 Radwag, Poland).

In order to minimize the variation of the results, for each time point, 8 different cultures were performed (4 samples were used to assess cellular parameters, and 4 to analyse cellulose-related properties). The appropriate order of individual analysis types described below e.g. weighing, water related parameters, IR spectra and degree of polymerization, allowed to use all 4 samples of the cellulose for each test.

Determination of BC production

The BC was harvested from the medium and weighed on an analytical balance (accuracy 0.0001 g, WTB 2000 Radwag, Poland). Subsequently, the BC was purified in 0.1 M NaOH / 80°C / 30 min. to remove bacterial cells and medium components and then rinsed with water. The NaOH treatment procedure was repeated three times. In the next step, the cellulose pellicle was dried in an oven (EV-50, Trade Raypa, Spain) at 60°C overnight, weighed again on an analytical balance and subjected to a series of analysis as described below.

Determination of water related properties

To perform the water swelling assessment, the cellulose pellicles were cut into 1 cm^2 samples, dried at 60°C for 6 h to remove any water content and weighed using an analytical balance. In the next step, the samples were immersed in distilled water till there was no further water absorption (approx. 1 h), wiped carefully with filter paper and weighed. The results are shown as percentage equilibrium degree of swelling (%SRE)[17] calculated using the following formula:

$$\%SRE = \frac{(W_\infty - W_{dry})}{W_{dry}} 100\% \quad (1)$$

where:
W_∞ is the weight of BC at swelling equilibrium BC and W_{dry} is the weight of the dry sample.

To determine the percentage of water release value (%WRV), the initial wet weights of the BC samples were measured on the analytical balance as stated above. Then the samples were continuously weighed during incubation at 25°C at different time intervals until a constant weight of the dry sample was achieved. The WRV value[18] was calculated from the equation (2):

$$\%WRV = \frac{(W_{dwet} - W_{dry})}{W_{dwet}} 100\% \quad (2)$$

where:

W_{dwet} is the weight of the swollen BC during drying and W_{dry} is the weight of the dry sample.

Degree of polymerization measurement

The degree of polymerization was determined by the measurement of viscosity of BC dissolved in 0.5 M solution of copper (II)-ethylenediamine (CUEN) using a capillary Ubbelohde viscometer (No. 1, Labit, Poland) as described by Tsouko et al.[19].

Culture medium characterization

At each time point, the pH was determined in the residual culture medium using a pH meter (Elmetron, Poland). The concentration of glucose in the culture medium was determined enzymatically using the Glucose Assay Kit (BioMaxima, Poland) according to the manufacturer's protocol. The results were expressed as a percentage of glucose remaining in the H-S medium during cultivation.

Quantification of *G. xylinus* cells

The growth rate of BC-producing bacteria was determined twice. For the first time in liquid H-S medium, after cellulose pellicle removal from the culture tube (cells in medium) and for the second time after digestion of the cellulose pellicle with cellulose (cells in cellulose). For the digestion, the cellulose pellicles were washed in distilled water, transferred to 5 mL of the solution of cellulase (Sigma-Aldrich, Germany) in 0.05 M citrate buffer (pH 4.8) and incubated with shaking for 24 h at 35°C. Then, the samples consisting of the culture medium or suspension obtained after digestion with cellulase were centrifuged for 20 min at 1500. The obtained pellets were washed in PBS (Phosphate Buffered Saline, Sigma-Aldrich, Germany), centrifuged at 1500 for 20 min and restored to the original volume with PBS.

The number of microorganisms was determined using AlamarBlue cell proliferation assay (ThermoFisher, USA). Two hundred μL of the obtained bacteria suspension was transferred into wells on a black 96-well microtitre plate (Becton Dickinson and Company, USA). Twenty μL of AlamarBlue were then added to each well. The plates were sealed and incubated for 1 h at 28°C. The fluorescence signal was measured using microplate fluorescence reader (Plate Reader AF2200, Eppendorf, USA) at wavelengths of 540 nm excitation and 590 nm emission.

Infrared spectroscopy of bacterial cellulose

The IR spectra of bacterial cellulose were analyzed by the Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) method, using ALPHA FT-IR Spectrometer (Burker Co., Germany). The spectra were collected in the range of 4000–400 cm^{-1}. For each BC sample, 64 scans of the resolution of 8 cm^{-1} were performed. The spectra were processed using the Omnic Software and normalized to 1 at 1160 cm^{-1} (COC stretching mode for glucose rings).

RESULTS AND DISCUSSION

As reported by Hesse and Kondo[20] the cellulose secreted is randomly deposited behind the microorganism to produce a membrane of a three-dimensional network. It has also been reported that the product formation within a static culture starts with the formation of island-

-like cellulose fragments on the broth surface. Next, the fragments close together to form a thin cellulose pellicle. The thickness of this layer increases with synthesis time to approx. 2–4 cm within 10 to 28 days. After this time no further significant increase in cellulose synthesis is observed[7]. Similar observations were also made in the current study. The first, thin cellulose layer was observed from the 3rd day of the cultivation, regardless of the *G. xylinus* strain, and this time point was determined as the beginning of our experiment. The mass of the cellulose increased constantly up to 15–17 days of cultivation (Fig. 1). After that time, for 3 strains, no further increase in BC weight was observed (Fig. 1 A, C, D). In contrast, the *G. xylinus* strain DSM 46602 synthetized cellulose for the entire time of the experiment, although after 15 days the synthesis became visibly slower (Fig. 1 B). As can be seen from Figure 1, the number of bacterial cells in the cellulose matrix increased along with the cellulose synthesis. It can also be noticed that bacterial growth continued for 6 days after the cellulose production stopped; however, reproduction rate visibly slowed down. After 21–23 days (depending on the bacterial strain) the growth slightly decreased and then remained at the same level until the end of the cultivation time. As reported by Hornung et al.[7], the floating cellulose pellicle becomes a barrier for the glucose mass transfer in the later stages of its synthesis. In contrast to the bacteria anchored to the cellulose fibrils, the growth rate of *G. xylinus* in a suspension increased rapidly to 7 days of the culture, and then decreased until 19–23 days (Fig. 1). After that time, the growth rate leveled up and remained at a similar level until the end of the experiment. It is well known that *G. xylinus* is an obligate aerobic microorganism[1]. The cellulose keeps the bacteria in close contact with the oxygen from the air. Therefore, the observation from the current study showing a declining trend of viable bacterial cells in the medium after 6 days of cultivation should be explained by the deficiency of oxygen resulting from the creation of the cellulose pellicle on the surface of the medium. As previously reported, formation of the cellulose pellicle prevents gas exchange between the medium and the atmosphere[7].

Glucose is the primary substrate for the synthesis of cellulose. It is incorporated in a 4-step metabolic pathway placed in cytosol and converted to UDP-glucose. In the next step, cellulose synthase located in the bacterial cell wall catalyzes the polymerization of UDP-glucose to poly β-1-4 glucan[21]. The process of polymerization of UDP-glucose to poly β-1-4 glucan can be disrupted by such environmental conditions as oxygenation, pH or temperature[22]. Glucose can also be readily converted to gluconic acid which significantly affects the pH of the medium. This may also affect the efficiency of the BC

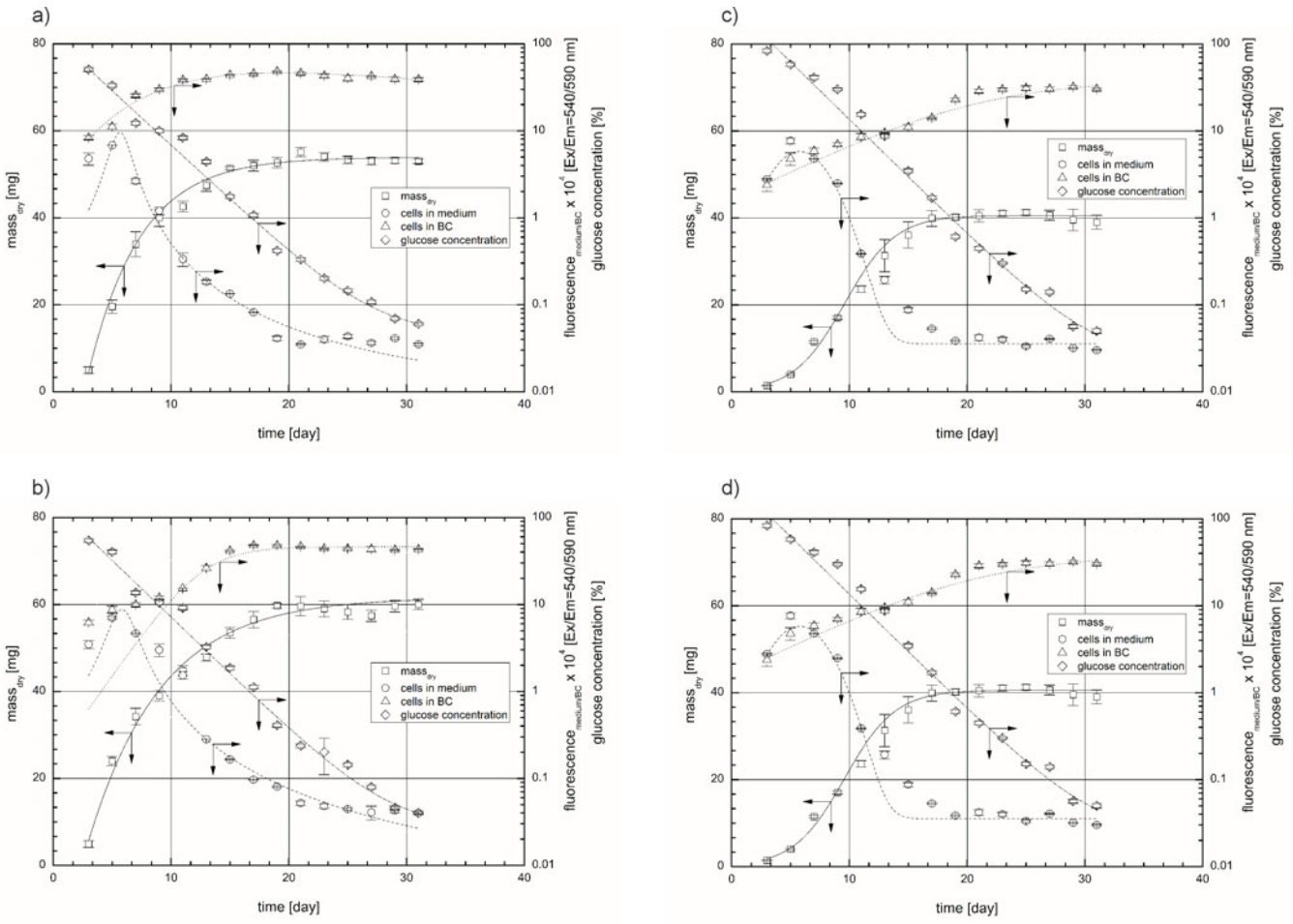

Figure 1. The rate of bacterial growth and cellulose synthesis and the changes in glucose concentration depending on the culture period and *G. xylinus* bacterial strain: A) ATCC 53524, B) DSM46602, C) DSM46604, D) DSM5067

$mass_{dry}$ – weight of cellulose after purification 0.1 M NaOH/80°C/30 min. and drying at 60°C overnight; cells in medium – number of bacterial cells in H-S medium based on the fluorescence signal of AlamaBlue reagent; cells in BC – number of bacterial cells in cellulose based on the fluorescence signal of AlamaBlue reagent; glucose concentration – percentage of glucose remained in H-S medium during cultivation.

synthesis process. The current study showed that glucose was almost entirely metabolized in the first 11–15 days of the cultivation (Fig. 1), i.e. in the period of cellulose biosynthesis. Such findings are consistent with the observation by Keshk and Sameshima[23] who reported that glucose was consumed rapidly in the early stage of incubation and almost completely (97%) after 7 days of incubation. Interestingly, in our study even 50% of glucose was metabolized within the time when the initial layer of BC could be observed (3 days). Earlier reports showed that insufficient substrate supply (of e.g. glucose) to the bacteria may be one of the most important external factors limiting the yield of the surface culture[7]. On the other hand, as reported by Toda et al.[24], in cultures where glucose is the primary carbon source, high concentration of gluconic acid can be another crucial factor responsible for the decreased synthesis of BC. The increased concentration of gluconic acid results in a decreased pH of the medium, which in turn reduces intracellular pH. As a consequence, high concentration of gluconic acid can change the activity of metabolic pathways responsible for the synthesis of BC[25]. As is widely accepted, optimum pH values range from 4.5 to 7.5 with the greatest efficiency being located around 6.5, whereas below the 3.5 value cellulose synthesis is inhibited[26, 27]. Although in our study glucose was metabolized relatively rapidly and the cellulose layer was formed as expected, in no case did the pH drop below the level which could inhibit cellulose synthesis (Fig. 2). However, based on the literature, changes in the pH value during the cellulose synthesis process may have a very complex character and thus it is difficult to clearly define the cause of the changes in this parameter. As an example, Yunoki et al.[28] did not observe any changes in the pH for seven days of cultivation of *G. xylinus* ATCC 10245. Park et al.[29] who cultivated the strain of *G. hansenii*

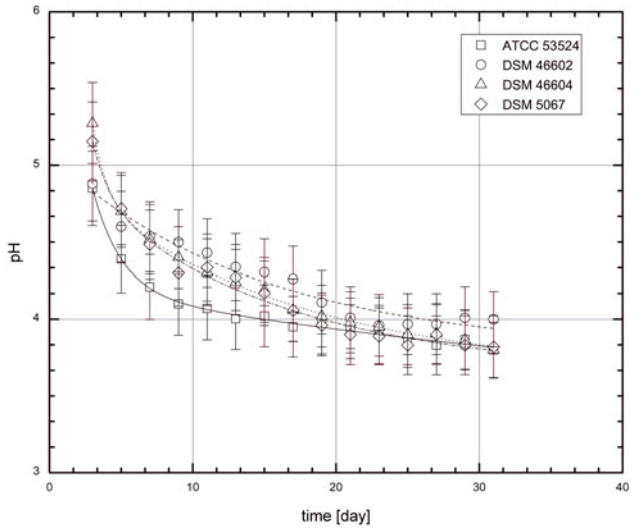

Figure 2. The pH of H-S medium depending on the culture period and *G. xylinus* bacterial strain

found a decrease in pH to 3 units on the 5th day of the culture. In contrast, in the study by Santos et al.[15] the pH dropped below 3.5 after the 4th day of incubation when glucose was used as a carbon source.

The cellulose synthetized by *G. xylinus* contains more than 90% of water[30]. It also displays a great capacity to absorb water. The wet mass of BC can be 100–200 times higher than its dry weight[31, 32]. The water resides inside the BC pores and is bound to the cellulose fibrils through hydrogen bonding[33]. The quantity of water trapped within the cellulosic scaffold depends on the strength of the physical adsorption on the fiber surface and on the density of reticulated fibers' linkage. The more space is available between the BC fibrils, the more water can penetrate and adsorb onto the material. Therefore, the difference in water capacity may be caused by the variances in fibril arrangement and porosity of various BC samples. The density of BC increases due to the secretion of more fibrils with the passage of time[34]. This in turn explains why the water-related properties of BC change along its synthesis time. The current study has shown that the BC obtained after 3 days displayed the highest water content as well as the highest water absorption capacity in comparison to the samples cultivated for a longer time (Fig. 3). Furthermore, cellulose pellicles obtained from cultures during consecutive days of cultivation, until the 15th–17th day, were characterized by a progressively decreasing water content as well as the ability to absorb water. The drop in the water swelling capacity of cellulose was especially well seen until the 11th day of culturing. Over the next 10 days, the water content value and the capacity to swell in water declined at a slower rate, reaching stabilization at approx. 21st–23rd day. Our observation agreed with the previous results reported by Al-Shamary and Al-Darwash[35] who demonstrated that the density of BC increased during the cultivation, whereas water capacity decreased due to the secretion of the fibrils with time. In the study by Sheykhnazaria et al.[16], SEM micrographs showed that the number of microfibril branches crossing to each other in the cellulose increased up to 14 days of its synthesis. However, further increase in synthesis time (up to 21 days) resulted in a decrease in the microfibril network.

It has also been demonstrated that the amount of water that escapes from the BC matrix to the environment depends on the arrangement of cellulose microfibrils[36]. The closely arranged microfibrils bind the water molecules more efficiently because of stronger hydrogen bonding interactions as compared to the loosely arranged microfibrils, which rather ineffectively protect the water from evaporation[37, 38]. In the current study, the values of water release obtained for BC from 3 strains declined until 11–17 days (depending on the strain) and then remained at the same level until the end of the experiment (Fig. 3 A, B, C). In contrast, in the case of the cellulose produced by the strain of *G. xylinus* DSM 5067, the water release value constantly decreased (Fig. 3 D).

The analysis of the polymerization degree of cellulose revealed that this parameter constantly decreased for the entire period of the cellulose synthesis (Fig. 4). As reported by Tahara et al.[39] the successive decrease in polymerization degree observed during cellulose synthesis can be related to the activity of the cellulase produced by most strains of *G. xylinus* cultivated in static culture conditions.

In light of the results obtained in the ATR-FTIR analysis, the spectra, regardless of the strain and time of BC synthesis, contained elements typical for BC (Fig. 5). From these analyses it can also be seen, that the intensity of bands in the individual spectral regions changes over the time of *G. xylinus* culture and BC synthesis. Most

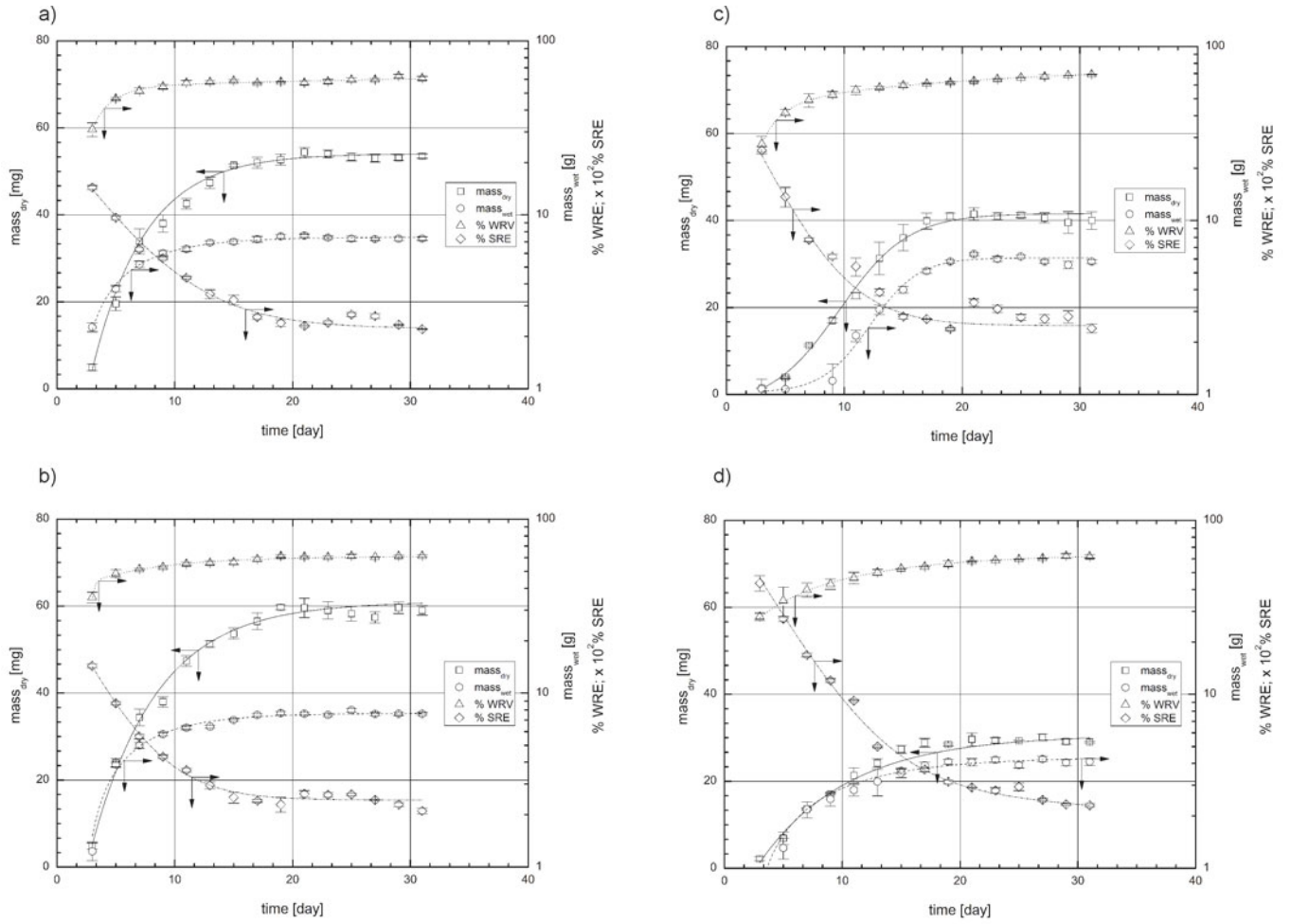

Figure 3. The dry mass and wet and water related parameters of cellulose depending on the culture period and *G. xylinus* bacterial strain: A) ATCC 53524, B) DSM46602, C) DSM46604, D) DSM5067
$mass_{dry}$ – weight of cellulose after purification 0.1 M NaOH/80°C/30 min. and drying at 60°C overnight; $mass_{wet}$ – weight of cellulose before purification and drying; %WRV – water release value; %SRE equilibrium degree of swelling.

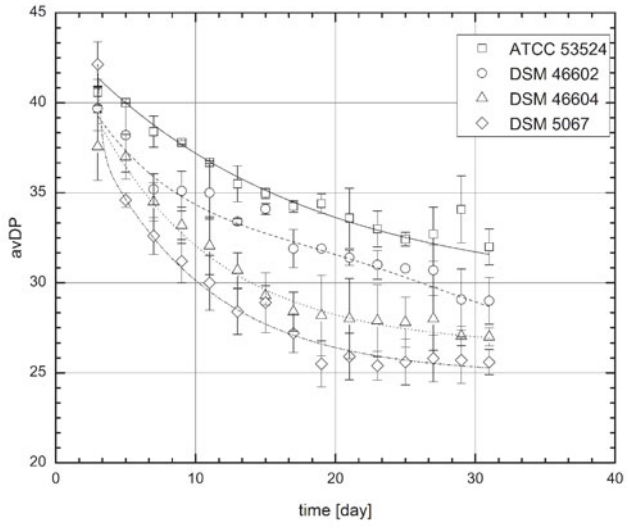

Figure 4. The polymerization degree of cellulose depending on the culture period and *G. xylinus* bacterial strain

likely, the changes refer to the progressive aggregation of cellulose microfibrils and the formation of an increased number of hydrogen bonds. In the range of spectrum from 1200 cm^{-1} to 800 cm^{-1} a difference in the intensity of the bands indicating the C-O group (primary and secondary alcohols) was observed. These changes indicate a progressive differentiation of the molecular structure related to the increase in the content of crystalline form and the decrease in amorphous state in cellulose. This is consistent with previous reports from Liu et al.[40] who reported that plant cellulose had a different ATR-FTIR profile depending on its age. The observed differences in BC molecular structure can be also explained by the drop in the values of the average degree of polymerization and may indicate changes in the size of crystallites over the time of BC synthesis.

Summarizing, the obtained results have shown that there is a general pattern for bacterial growth and cellulose synthesis conducted by various strains of *G. xylinus*. The most visible changes in the analyzed parameters of BC were observed within the first 10–14 days of the experiment. The general trend indicated that along with the culturing time the mass of the synthetized cellulose increased which correlated with the progressive increase in the number of bacteria attached to the cellulose matrix. The beginning of the formation of the cellulose pellicle was related to a decrease in the number of cells present in the suspension form in the culture medium. Active bacteria metabolized glucose which resulted in a constant reduction of this substrate as well as the pH value of the culture medium. The water properties also depended on the age of the BC samples. The parameters demonstrating the water capacity and ability to swell in water decreased from young to old cellulose, whereas the water release

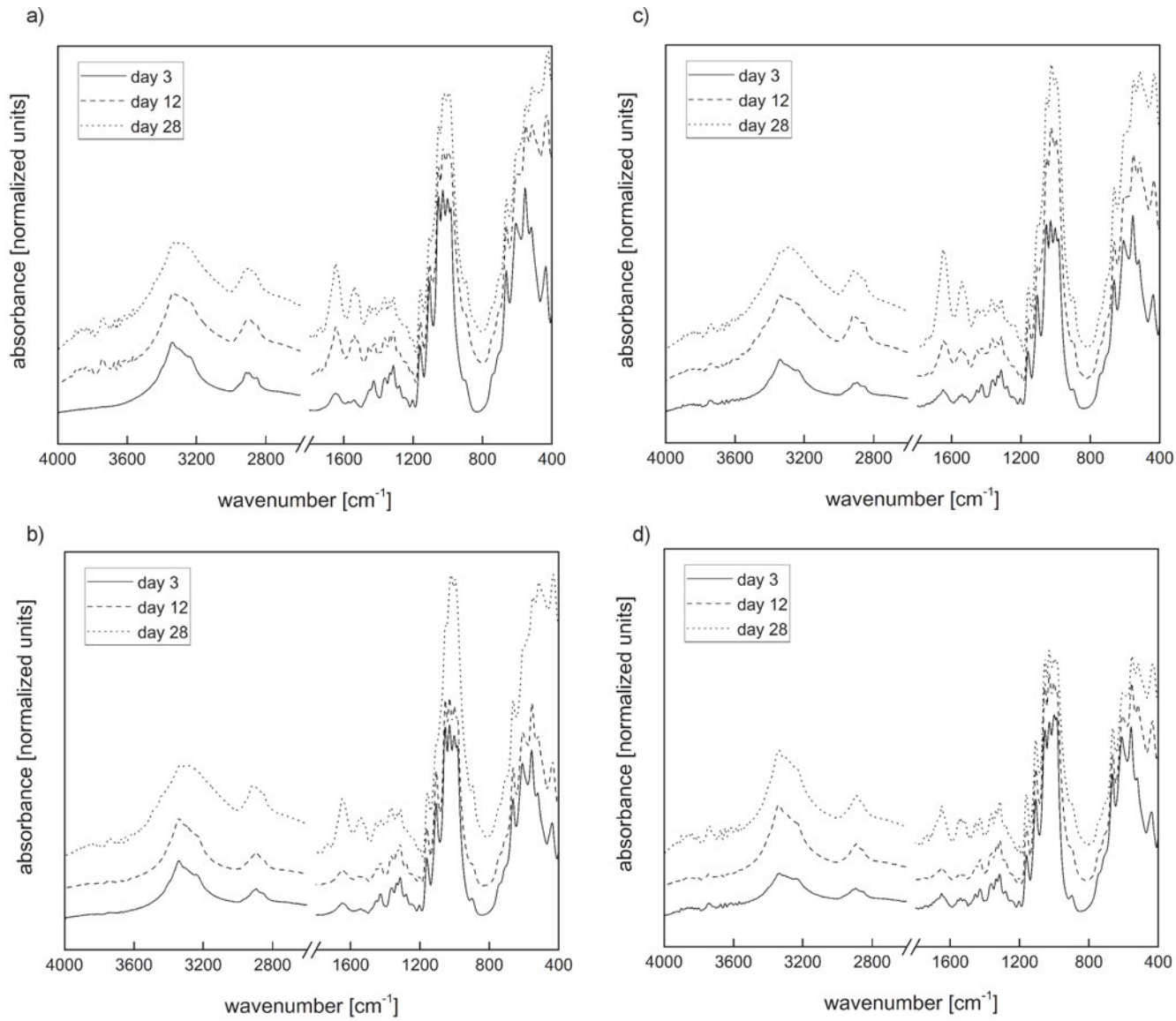

Figure 5. ATR-FTIR spectra of BC obtained after 3, 12, 28 days of synthesis for the individual bacterial strains: A) ATCC 53524, B) DSM46602, C) DSM46604, D) DSM5067

rate showed the opposite trend. Furthermore, the study showed that within the progressive cultivation time the BC polymerization value decreased. The results from the ATR-FTIR indicated a gradual differentiation of the molecular structure of BC depending on the content of crystalline and amorphous forms. It was also revealed, that among parameters showing dependence associated with the particular *G. xylinus* strain were the rate and period of BC synthesis, the growth rate of bacteria anchored to the cellulose fibrils, the capacity to absorb water and the water release rate.

The results obtained in the current study may be useful in the selection of optimum culturing conditions and period from the point of view of good efficiency of the cellulose synthesis process. They may also help obtain a material with the desired properties.

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