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Bacterial Cellulose Production by *Komagateibacter xylinus* with the Use of Enzyme-Degraded Oligo- and Polysaccharides as the Substrates

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Abstract: Bacterial cellulose (BC) is a unique biopolymer synthesised by many bacteria as a critical element of their biofilm matrix. The most known and efficient producers of BC are bacteria from the genus of *Komagataeibacter*. Bacterial cellulose, with its unique properties, high crystallinity, mechanical strength, and unprecedented ability to hold water, is an object of interest in many industries. Despite the enormous efforts that have been made to develop an effective process, the economic aspect of BC production is still a limiting factor for broadening applications, and new "breaking point" solutions are highly anticipated. In this study, the possibility of using sucrose, lactose, and starch as alternative carbon sources converted to simple sugars directly in the culture medium by microbial glycohydrolases, β-D-fructofuranosidase, β-galactosidase, and glucoamylase in the process of BC synthesis was analysed. The results showed the high potential of the enzyme-assisted fermentation process that, for most used raw carbons sources, was highly efficient, with a yield higher (i.e., lactose 40% more) or comparable to the cultures maintained on standard Hestrin-Schramm media with glucose as a sole carbon source. The X-ray diffraction, Fourier transform infrared spectroscopy, and scanning electron microscope analyses did not reveal any negative influence of enzyme-assisted cultivation on the BC material properties, such as crystallinity, swelling ratio, and moisture content. Applying specific enzymes for converting inaccessible, raw-form carbon sources to the culture medium of *Komagateibacter xylinus* opens a simple way to use various oligo- and polysaccharides acquired from many kinds of biomass sources in the BC production process.

Keywords: bacteria cellulose; hydrolases; oligosaccharides; biomass conversion; production

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1. Introduction

Cellulose is the most widespread biopolymer in the world. Although commonly most used is the one from plants, other organisms, such as algae, fungi, and bacteria, can produce it [1]. In structural terms, bacterial cellulose (BC) is compatible with that of plant origin, additionally being free from impurities in the form of lignin and hemicellulose. In the face of global environmental crises, including that caused by extensive deforestation, BC is an attractive alternative as a source of this biopolymer. Bacterial cellulose exhibits unique properties, such as complex nanostructure, high crystallinity, retention of large amounts of water, and high mechanical strength [2]. These material properties are the reasons for the high interest in developing BC-based composites and derivatives that could be applied in many fields, such as the electronics, food, and paper industries, as well as the medical

and veterinary fields [3,4]. Many species of bacteria have the ability to produce bacterial cellulose, including mainly *Acetobacter* spp., but also *Agrobacterium tumefaciens*, *Rhizobium* spp., and *Sarcina ventriculi* [5]. The most efficient BC producer is *Komagataeibacter xylinus*, an aerobic Gram-negative bacteria. The production of bacterial cellulose by these bacteria can be affected by various factors, i.e., the bacterial strain potential, cultivation method, carbon, nitrogen source, media acidity, or temperature [6,7]. BC synthesis by *K. xylinus* can be performed in a relatively wide pH range between 3.0–7.0 and a temperature of 25–30 °C. However, the optimal conditions depend on the specific requirements of the bacterial strain [8,9]. Despite many years of intensive research, the cost of cultivation media is still the most limiting factor for the wider use of BC in industry. Currently, the possibility of solving this issue is combined with using by-products and waste from the food industry and agricultural production as feedstocks to prepare cultivation media. Nowadays, the food industry generates a significant amount of waste that can be a valuable source of ingredients for cultivation media for *K. xylinus* [5,10]. For example, during sugar production by the processing of sugar cane or, on a smaller scale, sugar beets, significant amounts of molasses and pulp are produced [11,12]. Moreover, processing fruits, vegetables, and grains generates pomace containing significant amounts of various poly- and oligosaccharides [13,14]. In addition, the waste from the dairy industry, mainly whey, is also rich in various types of valuable oligosaccharides [15]. This biomass contains a significant number of saccharides, such as starch, sucrose, and lactose. However, due to the lack of appropriate metabolic pathways and enzymes, these poly- and oligosaccharides in the unchanged form are not available as carbon sources for *K. xylinus* cells in the BC synthesis process. The solution to this inconvenience may be the usage of different enzymes of microbial origin to liberate the potential of carbon sources from feedstocks considered waste materials, i.e., β-D-fructofuranosidase (β-D-Fru), β-galactosidase (β-Gal), and glucoamylase (GLA). β-D-Fru is an enzyme that hydrolyses sucrose to glucose and fructose. This glycohydrolase can be found in plants, fungi, and bacteria cells, which are important elements of many regulatory and signalling pathways [16–18]. In industry, this enzyme is mainly produced by *Saccharomyces cerevisiae* yeast. Invertase is an enzyme that has been used for years in various branches of the food industry, e.g., in the production of inverted sugar, artificial honey, alcoholic beverages, lactic acid, and glycerol. This enzyme has also been used in the pharmaceutical and cosmetic industries, and as an element of biosensors for sucrose detection [18]. The β-galactosidase of microbial origin is an enzyme that converts lactose into glucose and galactose. However, in specific conditions, such as water restriction in the reaction medium or saturation by hydrolysis reaction products, this enzyme is capable of transglycosylation reaction and synthesis of various galactooligosaccharides (GOS) [19]. β-Gal can be found in the organisms of young mammals, where it is responded to for degradation of the main milk disaccharide, lactose. In addition, it is also present in many fruits (apples, kiwi, tomatoes), where it plays along with other enzymes and substances an important role in softening the crumb and peel of fruits by removal of galactosyl residues from pectin and xyloglucan [20]. In food industrially, this enzyme is used primarily in dairy processing to produce milk with reduced lactose content, simplify the preparation of yoghurts and kefirs, or speed up solidification and maturation in short-term cheese production technology. Moreover, β-galactosidase is used for therapeutic purposes for people struggling with lactose intolerance in the form of dietary supplements. β-Gal is also used in producing prebiotic substances, such as galactooligosaccharides (GOS), an essential component of humanised substitute milk for newborns and infants [19]. Glucoamylase is a hydrolase belonging to the family of amylases (specifically, exoamylases) that catalyses the hydrolysis of starch through the breakdown of 1,4-α-glycosidic bonds between glucose subunits. The enzyme is produced by various microorganisms, from which glucoamylase produced by *Aspergillus niger* is widely used in many industries, e.g., distillery, brewing, baking, and biofuel production [21–23]. Apart from catalytic potential, the main advantage of the described enzymes is their availability as ready-to-use preparations with good purity and relatively low price.

This study aimed to analyse the influence of using different oligo- and polysaccharides converted directly in the cultivation medium by specific enzymes to accessible carbon sources and the effect of this solution on synthesis efficiency and selected material properties of BC.

2. Materials and Methods

2.1. Chemicals and Reagents

Enzymes, yeast β-D-fructofuranosidase (EC 3.2.1.26, *Saccharomyces cerevisiae*, VII grade), β-galactosidase (EC 3.2.1.23, *Kluyveromyces lactis*, Lactozyme 2600 L), and glucoamylase (EC 3.2.1.3, *Aspergillus niger*, AMG 300L), as well as reagents and components of the culture media used during the research, were purchased from Sigma-Aldrich with at least reagent grade purity and used without further purification. The strain of *K. xylinus* ATCC 53582 was ordered from American Type Culture Collection.

2.2. Preparation of the Culture Growth Media

The primary medium for *K. xylinus* culturing used in this study was a Hestrin-Schramm (HS) liquid medium consisting of yeast extract (2 g/L), bacteriological peptone (2 g/L), citric acid (1.15 g/L), Na_2HPO_4 (2.7 g/L), and $MgSO_4 \times 7\ H_2O$ (0.06 g/L), with glucose as carbon source (20 g/L). In the variants of tested media, the glucose was replaced with a poly- or oligosaccharide, such as sucrose, lactose, and starch, in the equivalent amount of 10 g/L. Moreover, control cultures were prepared: positive control, *K. xylinus* cultivated in a medium with 10g/L of glucose, and negative control, *K. xylinus* cultivated in a HS medium with test oligo- and polysaccharides as sole carbon source with omitting the enzymes addition. The experiment was set in three different batches for each tested medium and enzyme.

2.3. Bacterial Cellulose Synthesis and Purification

The primary culture solutions were prepared in 50 mL falcon-type tubes, in which the medium containing the bacterial cultures with the appropriate amount of the enzyme was mixed. Before transferring to cultivation media, enzyme solutions in sodium phosphate buffer (50 mmol/L, pH 7.0) were filtered using 0.45 µm syringe filters for sterilisation. The prepared cultures were poured into sterile culture vessels and covered on 24-well plates for cell culture with 2 mL of test and control culture medium in each cell (Figure 1). The culturing was conducted for 6 days at 28 °C. After incubation, BC pellicles were collected from the cultivation medium, rinsed in deionised water, and left overnight at 4 °C to remove the residual medium. Next, BC membranes were digested in 0.1 mmol/L sodium hydroxide solution in a water bath at 80 °C, for 3 cycles lasting 30 min each. Then, the digested BC samples were flushed in deionised water until the washing solution stabilised to pH 7.0. Finally, BC membranes were stored at 4 °C until further analysis [24].

2.4. Analysis of the Parameters of the Pre- and Post-Culture

2.4.1. pH Determination

The initial and remaining post-harvest media pH was determined using a laboratory pH meter (Elmetron, Zabrze, Poland).

2.4.2. Glucose Concentration Determination

Glucose concentration was determined by an enzymatic colorimetric method with glucose oxidase/peroxidase, using a commercial kit (GOD/PAP) according to the protocol supplied by the manufacturer (Biomaxima, Lublin, Poland). All measurements were conducted in triplicate.

2.4.3. Enzymes Activity Determination

The activity of enzymes was measured using buffered solutions of appropriate substrates dedicated to each enzyme: for β-D-fructofuranosidase, 180 mmol/L sucrose in

50mM acetate buffer, pH 4.7; for β-galactosidase, 80 mmol/L lactose in 50 mM phosphate buffer, pH 7.0; for glucoamylase, 10 g/L soluble starch in 50 mM acetate buffer, pH 5.0.

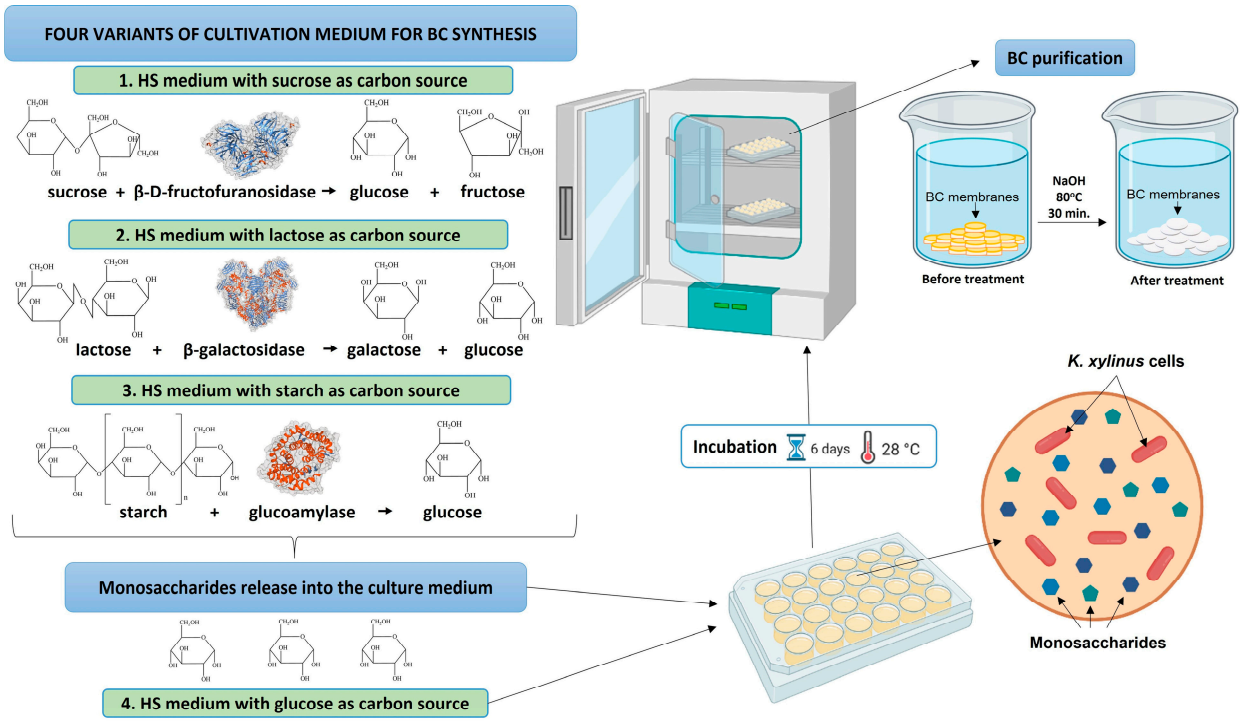

Figure 1. The scheme of the experimental *K. xylinus* cultivation process in the presence of a combination of oligo- and polysaccharides and specific enzymes.

The assays were performed with the use of 96-well microplates. In the beginning, 200 µL of substrate solution was equilibrated to the temperature of the assay 30 °C and transferred to wells on the microplate. Then, 10 µL of the solution with the given enzyme was added to the substrate and left for 10 min for β-D-fructofuranosidase, 15 min for β-galactosidase, and 5 min for glucoamylase. After the incubation, 10 µL of the reaction mixture was transferred to 200 µL of reagent for glucose concentration assay and left for 10 min at 25 °C. After 10 min, the absorbance was measured at 540 nm using a Tecan Infinite200Pro microplate reader. The glucose concentration was calculated using a 5.5 mmol/L standardised glucose solution. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µM of glucose per minute under the assay conditions.

2.5. Bacterial Cellulose Synthesis Efficiency and Yield Determination

Purified BC membranes were subjected to mass measurements of the so-called BC wet mass using an analytical balance (WTB,2000 Radwag, Radom, Poland), then dried at 60 °C and weighed again to obtain the value of the so-called BC dry mass. Next, the yield of cellulose production was calculated according to Equation (1). Afterward, based on the values of the obtained dry weight, the efficiency of the BC synthesis process was calculated according to Equation (2) [6]:

$$Yield\left(\frac{g}{l}\right) = \frac{m_d}{V_m} \quad (1)$$

where m_d—the dry weight of the obtained cellulose; V_m—the volume of cultivation media.

$$Synthesis\ efficiency\ (\%) = \frac{m_d}{m_t} \times 100\%, \quad (2)$$

where m_d—the dry weight of the obtained cellulose; m_t—total mass of the carbon source in the form of saccharide used as substrate.

2.6. Moisture Content Ratio and Swelling Ratio

The mass of thoroughly dried BC membranes was measured as mentioned above. Then the moisture content ratio was counted, as in Equation (3).

$$MCR\ (\%) = \frac{(m_w - m_d)}{m_d} \times 100\%, \quad (3)$$

where *MCR*—moisture content ratio; m_w—mass of swollen membranes; m_d—mass of the dry BC membranes (initial weight).

Samples from each experiment and control were prepared and placed in a cell on a 24-well plate. Each sample was then immersed in 2 mL of 100 mmol/L buffers at pH 4.0 and 5.0 (acetic buffer with the addition of sodium chloride) and pH 7.0 (buffer consisting of potassium hydrogen phosphate, sodium hydroxide, and sodium chloride), prepared according to [25], and then incubated at room temperature for 24 h. Swollen membranes were pulled out after 1, 2, 3, and 24 h intervals and weighed again (excess water was removed by gently pressing on the filter paper).

The resulting mass data were then determined as swellings ratio, which is counted according to Equation (4) [26]:

$$SR\ (\%) = \frac{(m_w - m_d)}{m_d} \times 100\%, \quad (4)$$

where *SR*—swelling ratio; m_w—mass of swollen membranes (g); m_d—mass of the dry BC membranes (initial weight; g).

2.7. BC Structural Properties Analysis

2.7.1. Attenuated Total Reflectance in Fourier Transformation InfraRed Spectroscopy (ATR-FTIR) Analysis

The previously dried samples of BC were analysed using an ATR-FTIR spectrophotometer ALPHA II (Bruker Co., Bremen, Germany). The spectra were collected in the range 4000–400 cm^{-1} with 32 scans and resolution of 4 cm^{-1}. The recorded ATR-FTIR spectra were further processed using SpectraGryph 1.2 software. The total crystallinity index (TCI) and lateral order index (LOI) were calculated from the ratio of bands absorbance value 1370 cm^{-1}/2900 $cm^{-1}$and 1430 cm^{-1}/895 cm^{-1}.

2.7.2. X-ray Diffraction Analysis (XRD)

The crystal structure of the BC was analysed by using a high-resolution X-Ray diffractometer (Aeris, Malvern Panalytical, Kassel, Germany) with CuKα radiation generated at 1.5406 *Å* wavelength, operated at 40 kV and 8 mA. The crystallinity index (*CrI*) of BC microfibrils was calculated using Equation (5)

$$CrI(\%) = \frac{(I_{200} - I_{am})}{I_{200}} \times 100\%, \quad (5)$$

where I_{200} is the maximum intensity of the peak corresponding to the plane in the sample with the Miller indices I_{200} at a 2θ angle between 21.5 and 24 degrees; I_{am} is the intensity of diffraction of the amorphous material at 18.3 2θ [27]. The crystallite size was calculated using the Scherrer Equation (6).

$$L = \frac{K \cdot \lambda}{\beta \cdot \cos\theta}, \quad (6)$$

where L is particle size (nm); K is the Scherrer constant (0.9); λ is the X-ray wavelength; θ is the Bragg angle; β is the full weight at half maximum (FWHM) of the peak from the fitting procedure in radians. The diffractograms were analysed using OriginPro2021 software.

2.7.3. Scanning Electron Microscope Analysis (SEM)

The micromorphology of BC was assessed using a Hitachi SU3500 scanning electron microscope. Prior to analysis, the purified, wet BC samples were dried overnight by lyophilisation in CHRIST Alpha 1–2 LD plus freeze dryer. Then, samples were sputtered with Cr using a SEM Quorum Q150T ES, a turbomolecular-pumped coater.

2.8. Statistical Analysis

The statistical analysis was performed using Statistica 14 software. Prior to analysis, the data were tested for the normality distribution using the Shapiro–Wilk test. The means were compared using one-way *ANOVA* and assessed by the Tukey post hoc test as significantly different at $p < 0.05$.

3. Results and Discussion

3.1. The Influence of Cultivation Media on Substrate Hydrolysis and Enzymes Stability

The possibility of BC production using alternative carbon sources other than glucose has been intensively researched for years [10,28–30]. These studies indicate that it is possible, but glucose is still the most widely used. However, it is worth remembering that bacteria use this monosaccharide in the biopolymer synthesis pathways, the development and growth of cellular biomass, or both [31]. Moreover, glucose is not an ideal carbon source for the synthesis of BC, and its usage is associated with the possibility of reducing the efficiency of the BC production process. The high concentration of this hexose in the culture medium causes its significant conversion, by oxidation, to gluconic acid (GA) by *K. xylinus* cells, which finally reduces the effectiveness of the BC synthesis process [32]. Moreover, an undesirable consequence of the overproduction of GA is acidification of the culture medium, which ultimately affects the bacterial metabolism and BC synthesis process. Nevertheless, using various monosaccharides as a carbon source in the cultivation medium allows for maintaining the efficiency of the BC production at a level that is impossible to achieve with different monosaccharide-rich oligo- and polysaccharides used as carbon sources [6,33]. Most characterised *K. xylinus* strains do not have the enzymatic potential for converting these kinds of carbon sources to simple sugars that then would be easily assimilated and transformed into precursors necessary for the synthesis of BC.

Due to the possible influence of medium components and parameters, such as pH or salinity, on enzyme activity and stability, the efficiency of substrate hydrolysis in time was analysed. The glucose liberation profile to media differed depending on the initial amount of used enzyme units. In the culture medium with sucrose and β-D-Fru, 6.0, 5.0, and 3.0 g/L of glucose were obtained after 4 h (Figure 2a). In the medium with lactose and β-galactosidase expected, 5.0 g/L of glucose was detected within 3 h for all variants of the enzyme units (Figure 2b). In the medium with starch and glucoamylase, glucose concentration was assumed within less than 4 h, with the highest speed in a medium containing 10 U/mL of the enzyme (Figure 2c). The profile of glucose liberation in media with sucrose and β-D-fructofuranosidase suggested the possibility of regulating the dynamics of increasing the accessibility of carbon sources. However, the ability of enzymes to act for an extended time depends on their stability in the reaction environment. The level of remaining activity, measured after finishing cultivation time, indicated that β-D-fructofuranosidase and glucoamylase retained similar initial activity levels, in contrast to β-galactosidase, which showed significantly lower operational stability (Table 1). The decreasing stability can be explained by the progressive decrease of medium pH that can negatively influence the enzyme's molecular structure. The acidic pH is especially not favourable for maintaining proper enzyme conformation and activity for a long time [34,35]. The profile of glucose liberation, particularly in media with sucrose and β-D-Fru, suggests the possibility of regulating the gradual increase of the accessibility of carbon sources by the amounts of used enzymes. It could potentially reduce the rate of media acidification and retain significant enzyme activity for a longer time. However, most initial substrates

were converted to monosaccharides in the first hour, when the number of bacterial cells was low and media pH was stable.

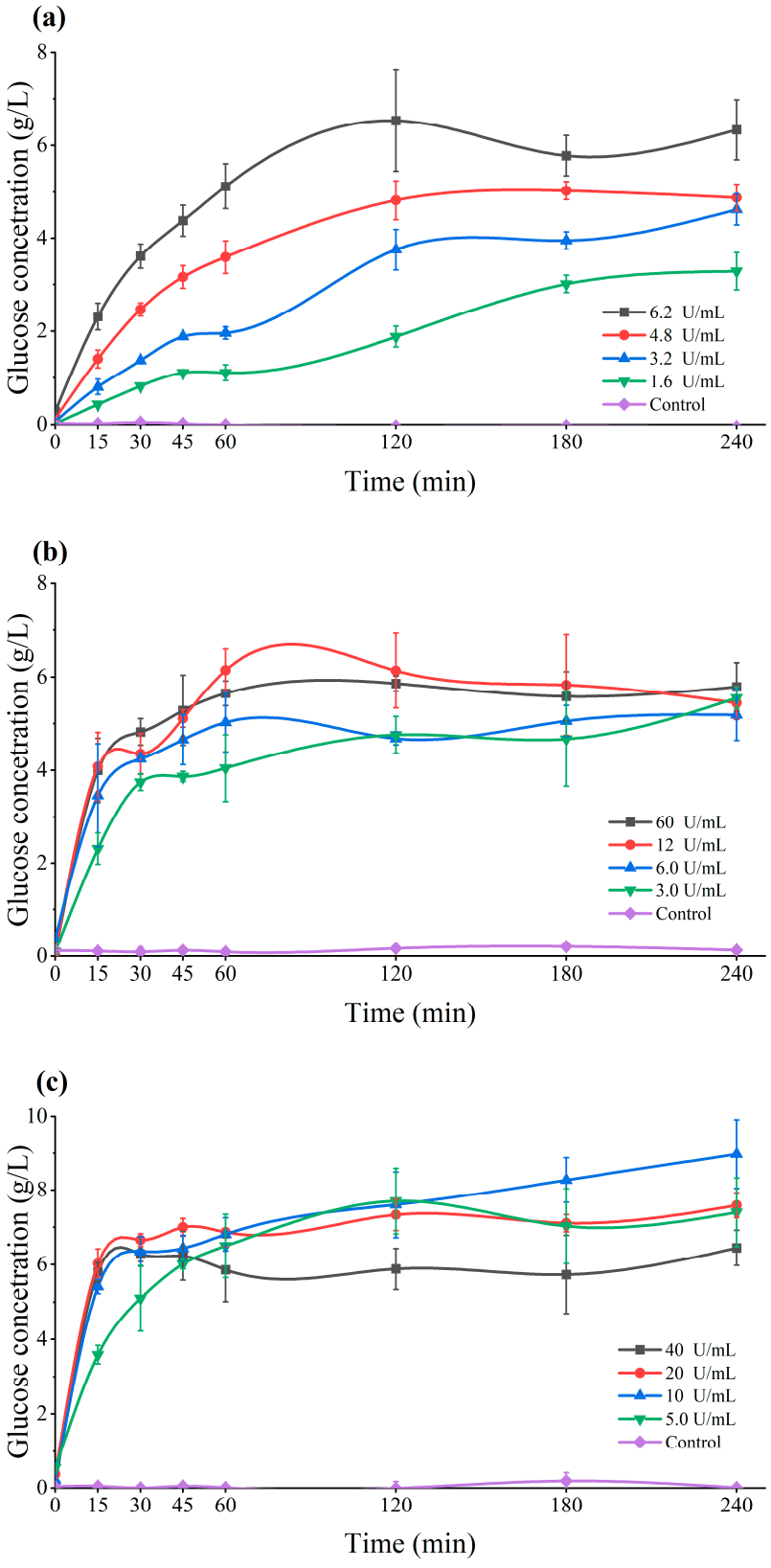

Figure 2. The effect of different amount units of β-D- Fru, β-Gal, and GLA on substrate conversion to simple sugars in HS medium with sucrose (**a**), lactose (**b**), and starch (**c**) as carbon sources.

Table 1. The influence of culture conditions on analysed enzymes stability.

Enzyme	Initial Amount of Enzyme in Medium (U/mL)	Remaining Activity * (%)
β-D-Fru	1.6	34.04 ± 14.35 ×
	3.2	29.50 ± 14.04
	4.8	38.05 ± 16.66
	6.2	48.20 ± 19.91
β-Gal	3.0	12.59 ± 9.43
	6.0	7.37 ± 5.19
	12	3.93 ± 2.07
	60	1.66 ± 1.90
GLA	5.0	14.15 ± 2.77
	10	23.60 ± 6.86
	20	32.69 ± 3.12
	40	50.96 ± 6.44

* The activity was measured in residual medium obtained after 6 days of *K. xylinus* cultivation. The medium's initial enzyme activity unit number was treated as 100%. × ±—standard deviation.

3.2. Yield and Synthesis Efficiency of BC

One of the most critical parameters of the cellulose production process is the efficiency of its synthesis. In the medium containing sucrose with hydrolysing disaccharide, yeast's β-D-fructofuranosidase, the yield and efficiency of biopolymer synthesis were comparable to standard HS media. Different enzyme unit numbers did not influence the yield, which amounted to approximately 4.0 g/L (Figure 3a). A significant quantity of BC was also obtained from the control cultures without enzyme addition, but with a much lower yield, 1.5 g/L, compared to standard HS and medium with the enzyme.

The BC synthesis efficiency was approximately 40% (Figure 3d), while for medium with enzymatically undegraded sucrose, approximately 7%. The obtained efficiency of BC synthesis was on a very good level, comparable to or better than earlier reports of the use of *K. xylinus* strain with a modified HS medium [6]. The second product of sucrose splitting by β-D-Fru is fructose, which can also be used as an effective carbon source for BC synthesis. However, its uptake by *K. xyllinus* cells and then use in BC synthesis requires additional energy for transportation across the cell membranes and conversion to glucopyranose. Therefore, bacterial cells use fructose as a secondary carbon source when glucose is present in a cultivation medium [36]. It is also worth highlighting the relatively significant amount of cellulose obtained from the control medium with sucrose and the omission of the β-D-Fru. It suggests that the analysed strain of *K. xylinus* may produce enzymes with β-D-fructofuranosidase activity. The available literature confirms the possibility of genes for these kinds of enzymes in some species from the genus Acetobacter sp., which partially confirms this hypothesis [37]. From the cultures cultivated in a medium with lactose and 12 U/mL of the β-Gal, the highest BC yield of 5.15 g/L was obtained. The cultures with the addition of 6.0 U/mL of enzyme resulted in 4.75 g/L for 3 U/mL; 3.83 g/L of BC yield was reached. Surprisingly, in the cultures with the highest amount of enzyme added (60 U/mL), a significant yield reduction was recorded, reaching 2.87 g/L (Figure 3b). Only a minor amount of BC was obtained in the control without enzyme, 0.2 g/L. The efficiency of BC synthesis by *K. xylinus* in the HS medium containing lactose and the enzyme β-Gal was also high. Cultures with 3, 6, and 12 U/mL of enzyme amounted to 38%, 48%, and even 52%, respectively (Figure 3e). It may be assumed that bacterial cells used most of the glucose released from lactose for BC synthesis. The high value of the BC synthesis effectiveness is astonishing, considering that the second product of lactose hydrolysis is galactose, and it cannot be included in the metabolism of both BC synthesis and biomass production [10]. Moreover, the lower acidification of cultivation media was not recorded, which could partially explain the high effectiveness. The cultures with the initial amount of enzyme at 60 U/mL were less effective in BC synthesis, with 27% efficiency (Figure 3e).

It may be a consequence of the specific properties of β-Gal and the ability to conduct transgalactosylation reaction, which can reduce the amount of available glucose in the reaction medium during the cultivation period of *K. xylinus* [38]. The lowest yield of BC production was found in the cultures of *K. xylinus* grown in a medium containing starch. The cultures cultivated with the addition of 5.0, 10, and 20 U/mL glucoamylase units obtained approximately 3.06, 3.23, and 3.11 g/L of BC, respectively. Cultures containing a higher amount of added enzyme, 40 U/mL, resulted in slightly lower amounts of CB, 2.41 g/L. In the control culture without enzyme, insignificant amounts of CB not exceeding 0.32 g/L were obtained (Figure 3c). Moreover, the macrostructure of obtained pellicles was highly disordered. The efficiency of BC synthesis in the cultures conducted in HS medium containing starch and glucoamylase was at a level not exceeding 31% for added enzyme amounts of 5.0, 10, and 20 U/mL. Significant lower efficiency was also observed for 40 U/mL, reaching only 24% (Figure 3f). The reason for this difference could be the faster decrease in the pH of the medium as a result of the faster release of glucose from starch, further oxidised to gluconic acid by *K. xylinus* cells. [33]. The high amount of GLA units allowed a short time to saturate the culture medium with glucose to the concentration, which caused its excessive oxidation to gluconic acid, which was manifested by the highest acidification of the medium and the limitation of the BC synthesis.

3.3. Attenuated Total Reflectance in Fourier Transformation InfraRed Spectroscopy (ATR-FTIR) and X-ray Diffraction (XRD) Analysis of Bacterial Cellulose

The ATR-FTIR and XRD were used for assessment of the influence of enzymes-assisted cultivation on the molecular BC properties. The ATR-FTIR spectra of BC obtained from *K. xylinus* cultures maintained in HS medium with various substrates and glycohydrolases are shown in Figure 4. In the range 800 cm^{-1} to 1800 cm^{-1}, all spectra contained a typical for BC set of bands with intense signals from adsorbed water at 1641 cm^{-1}, δCH2 (symmetric) of C-6 at 1427 cm^{-1}, δCOH in the plane from C-2 and C-3 at 1134 cm^{-1}, δOC of β-glycosidic linkage at 1160 cm^{-1}, δCO at C-6 at 1030 cm^{-1}, and δCOC of β-glycosidic bonds of amorphous BC phase at 897 cm^{-1}. In the second part of spectra at range from 2600 cm^{-1} to 3600 cm^{-1} were seen the bands specific for δOH for hydrogen and covalent bonds at 3334 cm^{-1} and δCH at 2895 cm^{-1} [24]. The ATR-FTIR spectra analysis showed no presence of significant changes in bands intensivity that could point at the modification of the BC molecular structure by the action of the analysed enzymes and used as carbon sources, oligo- and polysaccharides. The impact on the BC synthesis process at the cellular level or alteration of BC microfibrils structure by constituents added to the cultivation media can be demonstrated by alteration of BC crystallinity and porosity [39]. Nevertheless, the IR crystallinity index's values (LOI, TCI) did not differ considerably between the BC samples obtained from cultivation media with different enzymes and substrates (Table 2).

The XRD analysis was consistent with ATR-FTIR and showed that the addition of enzymes into cultivation media has no influence on BC microfibrils' molecular structure (Figure 4b). The crystallite sizes of BC from control cultures and with the addition of enzymes ranged from 5.4 nm to 5.9 nm, consistent with earlier reported values [36]. Moreover, no significant differences in the crystallinity (CrI) of BC microfibrils were detected, which indicates the neutrality of the used glycohydrolases for the BC synthesis process.

3.4. Scanning Electron Microscope (SEM) Analysis

SEM imaging allows for determining the structure information of the obtained biopolymer. The results of the analysis of the fibre parameters from SEM imaging are presented in Figure 5. As can be seen, every tested BC sample reveals its characteristic net-like 3D structure with high density and porosity between the fibrils simultaneously (Figure 5a–k).

The microfibres' arrangement was essentially random. The diameters of the fibrils were similar, mainly in the range of about 39.13 nm (±9.28 nm) (Figure 5e,f) from a medium with sucrose and β-D-Fru, 41.30 nm (±10.28 nm) (Figure 5h,i) in a medium with lactose and β-Gal, and 40.19 nm (±10.82 nm) (Figure 5k,l) obtained from starch and GLA medium

and about 40.46 nm (±9.80 nm) (Figure 5b,c) from standard HS (the mean values do not differ significantly between the different sample trials ($p < 0.005$). These values are in line with the literature [40]. As a result of the analysis, it can be concluded that, regardless of the culture medium used, the structure of BC was not affected. Moreover, the diameters or porosity of the membranes were similar to control samples, which was also confirmed by the visual assessment of the samples.

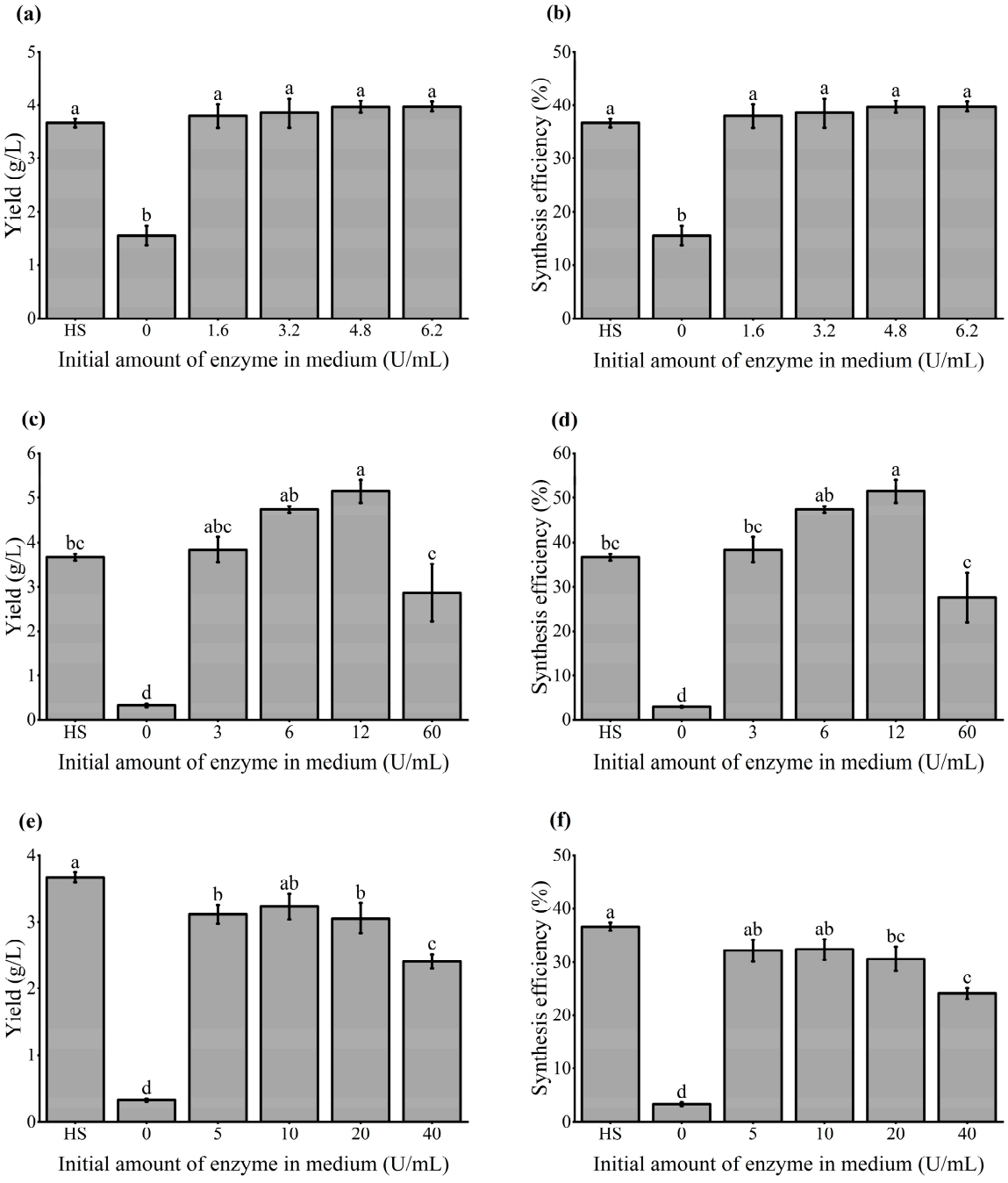

Figure 3. BC synthesis yield and efficiency for samples growing on medium containing sucrose (**a**,**b**), lactose (**c**,**d**), and starch (**e**,**f**), supplemented accordingly with β-D-Fru, β-Gal, and GLA in proper units' concentration. Bars marked as HS refers to parameters of *K. xylinus* cultures maintained on standard Hestrin-Schram medium with glucose as the sole carbon source. The means with common superscript are not significantly different from each other ($p < 0.05$). Error bars represent standard deviation.

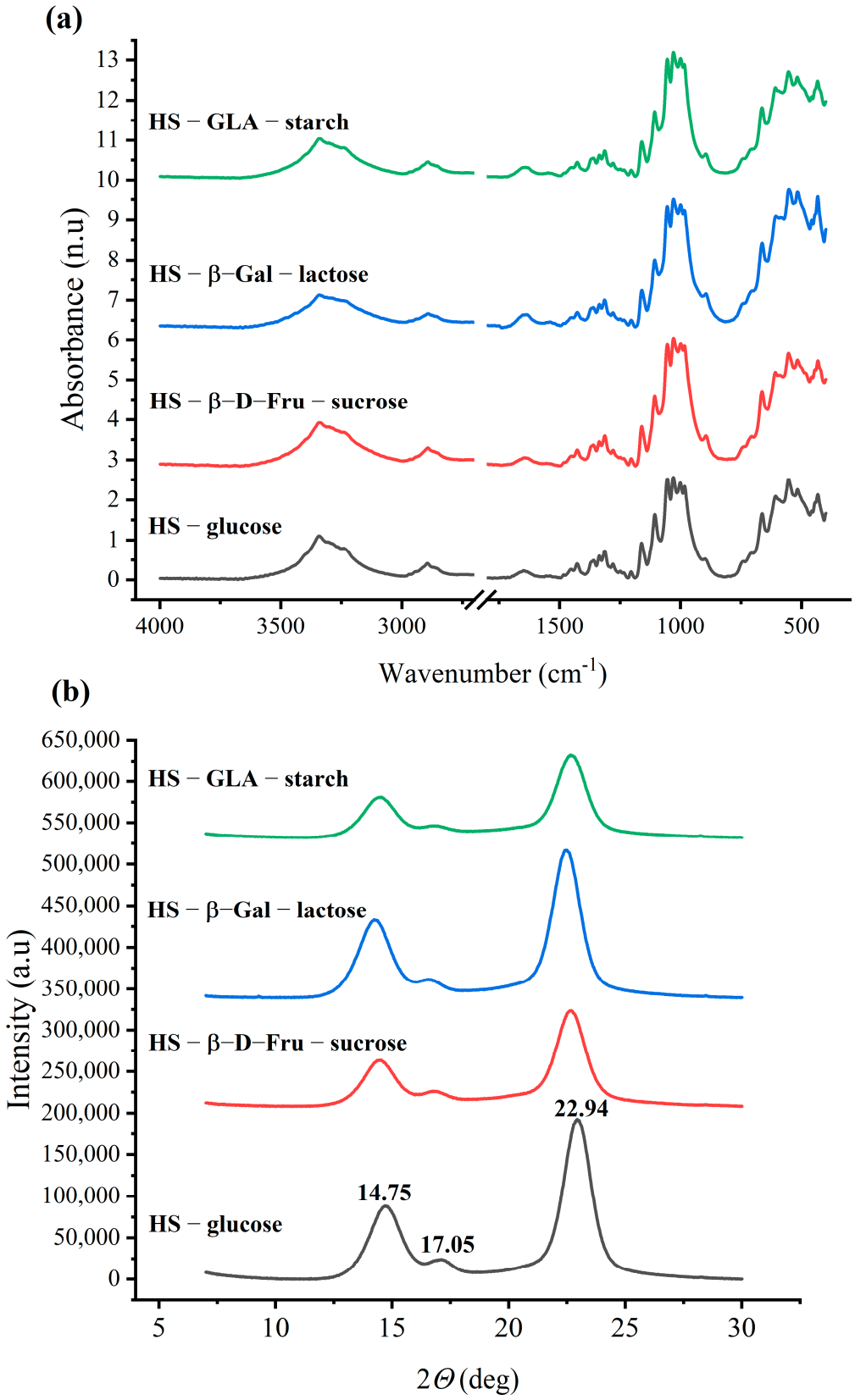

Figure 4. The representative ATR-FTIR spectra (**a**) and XRD diffractograms (**b**) of bacterial cellulose obtained from cultures with enzymatic conversion of sucrose, lactose, and starch to accessible carbon sources. The spectra from analysis BC samples obtained from modified media with the highest enzyme units amounts.

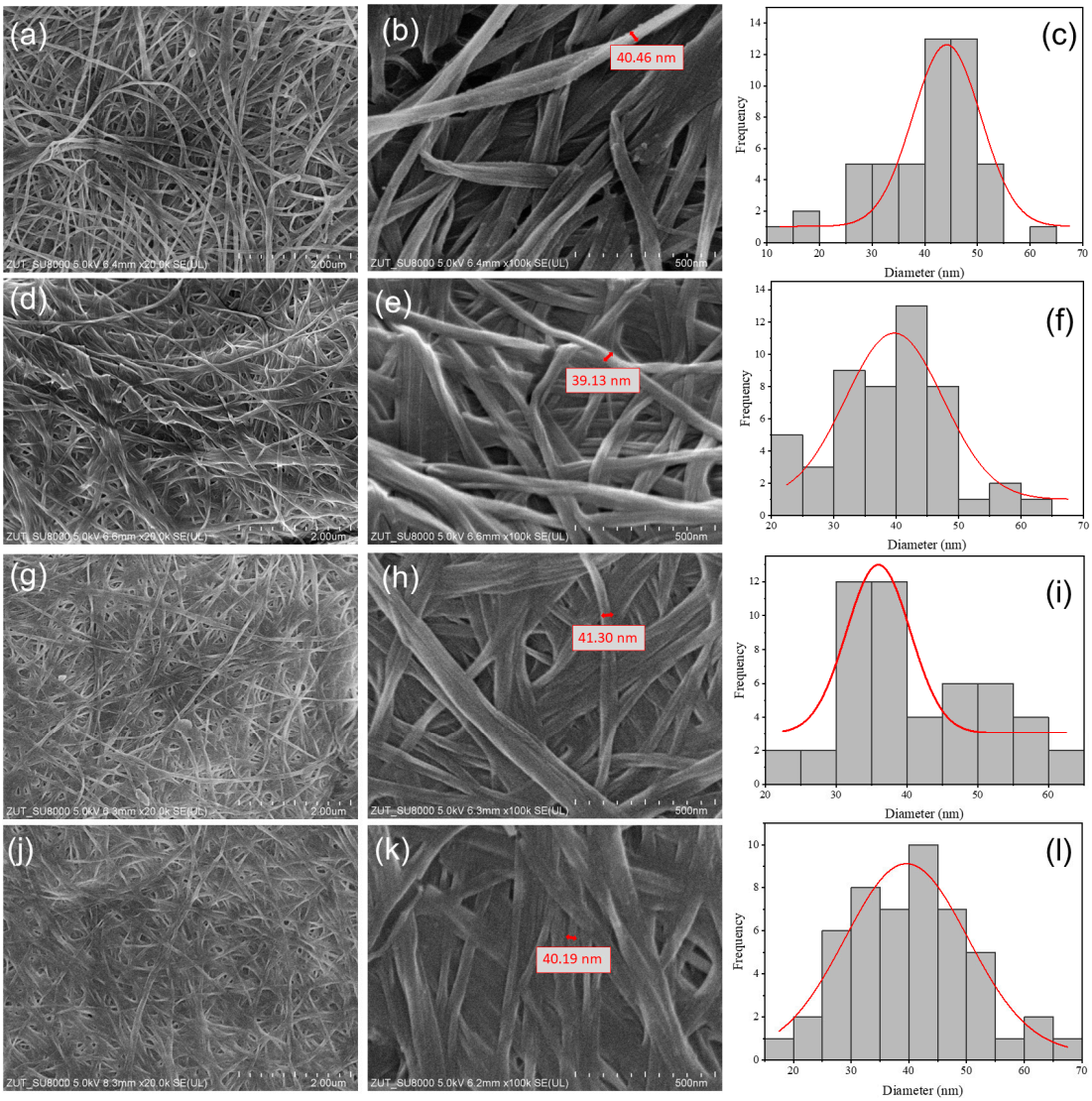

Figure 5. The SEM images of the BC obtained from cultures with different oligo- and polysaccharides as carbon source hydrolysed by specific enzymes at 20,000× (left column), 100,000× (centre column) magnification and distribution of BC microfibrils diameters' (right column). The BC samples obtained from standard HS medium—(**a**–**c**), medium with sucrose and β-D-Fru—(**d**–**f**), medium with lactose and β-Gal—(**g**–**i**), and from a medium with starch and GLA—(**j**–**l**). The presented SEM images show the BC surface obtained from modified cultivation media with the highest enzymes units amounts.

3.5. Moisture Content and Swelling Ratios

The moisture content ratio of BC pellicles is the most crucial parameter concerning their further application in medicine or cosmetology. It can be modified by many factors, such as the properties of the cultivation media and the potential of the used *K. xylinus* strain [41]. The appropriate moisture content of dressing materials strongly determines the wound-healing process's success and inhibits infectious organisms' colonisation [42]. Depending on the cultivation medium variant, the final mass of BC without water (which corresponds to the mass of the cellulose fibres only) amounted to less than 2% of the initial wet BC weight that resulted in obtaining the MCR on a 98% level or higher (Table 3). The values were consistent with the other earlier reports for BC produced in various fermentation systems [43,44].

The second water-related tested parameter of BC was the swelling ratio, which is also an essential property of this biopolymer. The value of this factor strongly depends on

the pH and ionic strength of the solvent. The swelling occurs due to increased distance between the chains in the polymer structure, which induces the release or absorption of water molecules or any other solution in which the BC was wetted [45]. The obtained BC, regardless of the medium variant used for their *K. xylinus* cultivation, exhibited a similar speed of maximum water uptake, regardless of the solution's pH, achieved after an hour of incubation, without significant changes after a further 24 h (Figure 6). Nevertheless, the BC from cultures with enzymes combined with oligo- and polysaccharide exhibited the highest SR at pH 5.0 (Figure 6b,e,h). In comparison, the BC from standard HS media showed the best SR at pH 7.0 (Figure 6c,f,i). High water retention is one of the most important and unique advantages of BC and its applications. It is worth noting that rehydration of once-dried BC does not allow obtaining such a high degree of biopolymer hydration, and the water content is about a few percent of the initial wet polymer mass and depends on the ionic strength of the solvent and the humidity condition of the experimental environment [46,47].

Table 2. The crystallinity degree and crystallite size, LOI, and TCI index values for BC obtained from different variants of *K. xylinus* cultures.

Sample	Initial Amount of Enzyme in Medium (U/mL)	XRD		ATR-FTIR	
		CrI (%)	Crystallites Size (nm)	LOI	TCI
Control	—	93.6	5.9	1.75 ± * 0.095	1.46 ± 0.10
β-D-Fru	1.6	95.1	5.9	2.04 ± 0.64	1.49 ± 0.31
	3.2	92.4	5.4	1.80 ± 0.38	1.46 ± 0.18
	4.8	90.1	5.6	1.89 ± 0.51	1.37 ± 0.07
	6.2	91.8	5.6	1.79 ± 0.37	1.33 ± 0.15
β-Gal	3.0	91.6	5.6	2.17 ± 0.42	1.53 ± 0.23
	6.0	91.6	5.6	2.17 ± 0.48	1.74 ± 0.14
	12	94.1	5.5	2.12 ± 0.40	1.63 ± 0.14
	60	94.2	5.8	2.06± 0.34	2.23 ± 0.19
GLA	5.0	93.9	5.9	1.59 ± 0.26	1.23 ± 0.12
	10	90.9	5.7	1.69 ± 0.23	1.35 ± 0.12
	20	91.8	5.6	1.80 ± 0.19	1.39 ± 0.08
	40	91.2	5.5	1.62 ± 0.35	1.44 ± 0.12

* ±—standard deviation.

Table 3. The values of the moisture content ratio of BC depend on the initial number of enzyme units and the kind of polysaccharides used as carbon sources.

Enzyme	Initial Amount in the Medium (U/mL)	Moisture Content Ratio (%)
Control	—	98.99 ± * 0.08
β-D-Fru	1.6	98.92 ± 0.13
	3.2	99.00 ± 0.10
	4.8	99.01 ± 0.06
	6.2	98.97 ± 0.06
β-Gal	3.0	99.01 ± 0.17
	6.0	99.07 ± 0.13
	12	98.96 ± 0.12
	60	98.62 ± 0.45
GLA	5.0	98.83 ± 0.13
	10	98.96 ± 0.16
	20	99.02 ± 0.18
	40	99.05 ± 0.19

* Standard deviation.

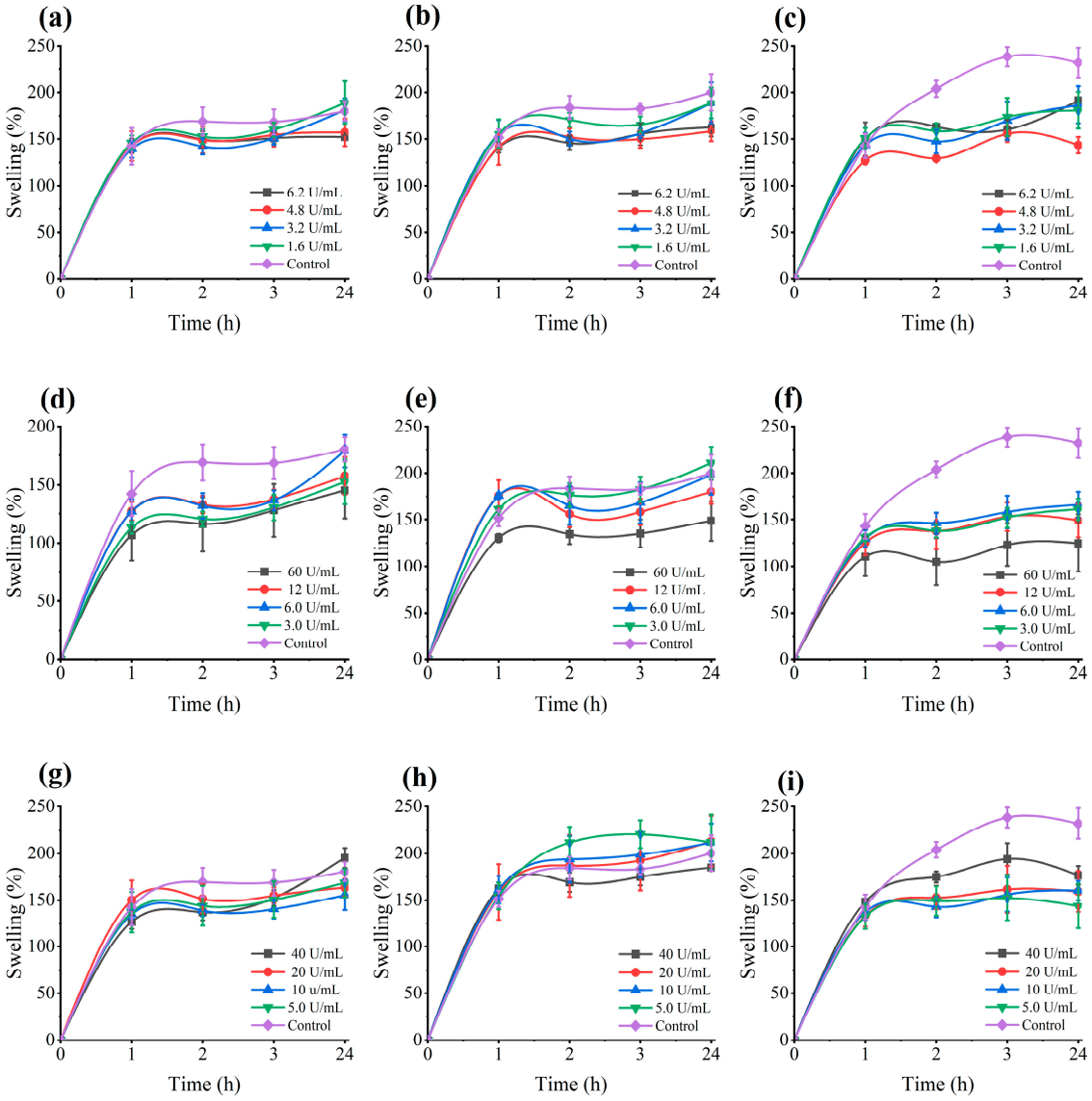

Figure 6. The influence of pH on the swelling ratio of BC obtained from analyses on cultivation media variant. The SR for BC obtained from the medium with sucrose and β-D-Fru at pH 4.0 (**a**), 5.0 (**b**) and 7.0 (**c**), lactose and β-Gal at pH 4.0 (**d**), 5.0 (**e**), and 7.0 (**f**); with starch and GLA at pH 4.0 (**g**), 5.0 (**h**), and 7.0 (**i**).

4. Conclusions

The liberation of the potential of inaccessible-in-raw-form oligo- and polysaccharides by use of specific glycohydrolases as an effective alternative carbon source to produce bacterial cellulose was successfully developed. The engagement of readily available and cheap industrial enzymes to degrade the glucose-rich saccharides that are widely available as waste materials of the food industry significantly improves the economic aspect of BC production. Apart from the tested oligo-and polysaccharides, considering the number of commercially available preparations of glycohydrolases, the results encourage using other carbon sources. Our study showed that the action of enzymes was not significantly affected by media components or acidification, and even a low amount of used enzyme was satisfactory for the effective conversion of initial substrates. The BC produced in the presence of specific enzymes presented good growth yield and efficiency, high crystallinity, high water contents, and significant swelling properties. The promising results of our study suggest that the next step should be the analysis of the possibility of using enzymes to treat waste from the dairy, fruit, and vegetable processing industries to further its utility as

substitutional nutrient ingredients for BC production. Compared to pure carbon sources, these substrates are not homogeneous and can contain much debris. These factors can also influence bacterial cells and enzymes, finally determining the BC synthesis efficiency. However, to confirm this hypothesis, further research is needed.

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