Cellulose (2023) 30:10811–10824
https://doi.org/10.1007/s10570-023-05559-0

ORIGINAL RESEARCH

Characterization of bacterial cellulose produced by *Komagataeibacter xylinus* strains grown in styrene/glucose mixtures

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Received: 22 April 2023 / Accepted: 11 October 2023 / Published online: 26 October 2023

Abstract This study is focused on the characterization of bacterial cellulose (BC) produced by *Komagataeibacter xylinus* strains DSM 2325, DSM 2004, and DSM 46604 from styrene/glucose mixtures. Styrene, the aromatic monomer of petrochemical plastics such as polystyrene, served as a co-substrate for bacterial cultivation, being assimilated by all strains, although with differing efficiency for BC biosynthesis. The best performing strain was *K. xylinus* DSM 2325 with a BC production of 2.70 ± 0.4 g/L. Interestingly, *K. xylinus* DSM 2004 produced BC from styrene as the sole carbon source, yielding 0.32 ± 0.02 g/L BC. The presence of styrene in the cultivation media had a minor influence on the produced BC chemical structure, thermal degradation temperature (318–337 °C), and morphology, where compact fibers of diameters ranging from 31 to 47 nm were observed. The crystallinity index of the samples was obtained through X-ray diffraction and showed that values varied according to the medium used (41–33%). However, the membranes synthesized in the presence of styrene were thinner (3–22 µm) than those produced from glucose (12–44 µm) and had low gas permeability. *K. xylinus* DSM 2325 and DSM 2004 membranes had also low permeability for O_2 (1.1–2.5 barrer) and CO_2 (2.5–5.8 barrer), while those produced by *K. xylinus* DSM 46604 had a higher permeability to CO_2 (42.3 barrer) together with low permeability to O_2 (2.5 barrer). Moreover, BC produced by *K. xylinus* DSM 2325 with styrene as an additive showed the highest crystallinity among all strains and mediums (46%). These results show the feasibility of using styrene as an effective co-substrate in a sustainable approach for its valorisation into a value-added biopolymer, with the advantage of tuning BC properties according to the envisaged application, by selecting the appropriate producing strain and culture medium.

Keywords Bacterial cellulose · *Komagataeibacter xylinus* · Styrene · Glucose

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10570-023-05559-0.

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Introduction

Bacterial cellulose (BC) is a biopolymer composed of covalently linked glucose residues held together by hydrogen bonds between carbon atoms 1 and 4, β-(1,4) linkages (Cacicedo et al. 2016). Production of BC is carried out through oxidative fermentation by bacteria from the genera *Komagataeibacter, Sarcina, and Agrobacterium* (Aswini et al. 2020), offering the advantage over plant cellulose of not being associated with lignin or hemicelluloses, as their removal is pricy and polluting (Naomi et al. 2020). BC comprises a fibrous network nanostructure with a large surface area and considerable porosity (Mishra et al. 2022) standing out among other biomaterials due to its unique characteristics, such as a high degree of polymerization (Mishra et al. 2022), biocompatibility (Portela et al. 2019), biodegradability (Torgbo and Sukyai 2020), high crystallinity (35–96%) (Andritsou et al. 2018; Gorgieva and Trček 2019), and excelling mechanical performance, exhibiting tensile strength values in the range of 200–300 MPa and Young's modulus up to 15–35 GPa (Cacicedo et al. 2016). Moreover, it presents superior water absorption and retaining abilities (around 99%) (Rebelo et al. 2018) due to its native hydrogel-like structure. These properties grant BC the versatility of being suitable for a wide range of applications, including cosmetics, food, textile, and biomedicine (Cacicedo et al. 2016; Zhong 2020; Mishra et al. 2022).

BC production is mainly carried out by either static or agitated cultivation, each strategy resulting in different material properties and morphology (Pang et al. 2020). Under static conditions, a gelatinous membrane is formed at the air–liquid interface of the culture medium and the process usually has higher BC production yields (Chen et al. 2018). Agitated cultivation generates small irregular BC pellet suspensions, and, although being a more scalable process, it shows lower yields due to the induction of non-cellulose producing mutants (Zhong 2020). Regardless of cultivation mode, the choice of culture medium is crucial to ensure bacterial cell growth and BC production, severely impacting polymer structure and physical properties. Glucose, sucrose, fructose, mannitol, arabitol, and molasses are common carbon sources reported for BC production, while yeast extract and peptone are preferred nitrogenous sources (Buldum et al. 2018; Singhsa et al. 2018; Andriani et al. 2020). Moreover, some agricultural and/or industrial wastes and by-products have been proposed as cost-effective alternative feedstocks to the refined carbon sources for BC production costs reduction, including but not limited to, wheat straw acid hydrolysate (Hong et al. 2011), whey (Revin et al. 2018), and industrial wastewater (Li et al. 2015). Other substrates and co-substrates have also been tested for BC production, e.g., aromatic compounds such as terephthalic acid (Esmail et al. 2022) and naphthalene (Marín et al. 2019), as well as alcohols (Lu et al. 2011) and organic acids (Lee et al. 2011). Although some improvement in BC biosynthesis has been achieved by using non-synthetic carbon sources, there is still a need to find other more efficient and scalable options.

Styrene is a mono-aromatic compound commonly used in the synthesis of petrochemical plastics, e.g., polystyrene (PS), acrylonitrile butadiene styrene (ABS), and styrene acrylonitrile (SAN) (Aliabadi et al. 2012). Although those materials are highly versatile and cheap to produce, and therefore used on a large scale, they are non-biodegradable, accumulating in landfills and marine ecosystems when not properly discarded, posing a threat to our environment, as well as our health, since styrene has been associated with the depression of the central nervous system, damage to the liver, hormonal disruptions, and cancer (Tan et al. 2015; Hwang et al. 2020). Thus, it is paramount to develop strategies that allow the elimination of styrene-laden effluents and styrene-based solid wastes from our environment. A promising prospect is its bioconversion into less harmful compounds, where microorganisms are employed as biocatalysts. Some efforts have been made to isolate bacterial strains that can feed on styrene and produce value-added compounds, such as poly(hydroxyalkanoate) (PHA) (Tan et al. 2015; Alonso-Campos et al. 2022) and phenylacetic acid (PAA) (Ebciba et al. 2020). However, to our knowledge, no studies focused on the biosynthesis of BC from styrene.

In this work, three bacterial strains, namely *Komagataeibacter xylinus* DSM 2325, DSM 2004, and DSM 46604, were cultivated using styrene as a co-substrate in styrene/glucose mixtures to assess their ability to biosynthesize BC and the impact of such cultivation conditions on the physical–chemical properties of the produced biopolymer. The resulting BC membranes were characterized in terms of their

morphology, chemical structure, crystallinity, thermal and mechanical properties, and gas permeability.

Materials and methods

Bacterial cellulose production

Microorganisms and media

Komagataeibacter xylinus strains DSM 2325, DSM 2004, and DSM 46604 were used for BC production. Hestrin-Schramm (HS) medium (Hestrin and Schramm 1954) (per litre: glucose, 20 g; peptone, 5 g; yeast extract, 5 g; citric acid, 1.15 g; disodium hydrogen phosphate, 2.7 g; pH = 7) was used for all experiments. HS medium was supplemented with styrene (0.5 g/L) (synthesis grade, Sigma-Aldrich) as a co-substrate. Experiments were also conducted with HS medium devoid of glucose and with styrene as the sole carbon source.

Production assays

The inocula for the experiments were prepared by inoculation of 1 mL of the cryopreserved cultures in 100 mL of HS medium (20 g/L glucose) and incubation in an orbital shaker, at 30 ℃ and 150 rpm, for 24 h. The assays were conducted under static conditions, in T-75 flasks (BIOFIL) containing 30 mL medium, at 30 ℃, for 18 days. A 20% (v/v) inoculum prepared as described above was used in each assay. At the end of the assays, the BC membranes were collected from the flasks and treated with 0.1 N NaOH, at 80 ℃, for 20 min. After washing with deionized water in an orbital shaker for 48 h (200 rpm, at room temperature) (Esmail et al. 2022), the membranes were lyophilized (ScanVac CoolSafeTM, LaboGene) at −110 °C for 48 h. Wet BC pellicles were weighed after alkaline treatment, as well as after lyophilization, for BC gravimetric quantification.

Analytical techniques

Broth samples (10 mL) were collected at the beginning and the end of the assays, centrifuged (10,956 g, 15 min, 4 ℃), and used for glucose and styrene quantification. For glucose quantification, the cell-free supernatant was diluted in H_2SO_4 0.01 N and filtered with modified nylon centrifugal filters (0.2 µm, VWR), at 10,000*g* for 10 min. Glucose concentration was determined by HPLC with a VARIAN Metacarb column (BioRad) coupled to a refractive index (RI) detector, as described by Rebocho et al. (2019). The analyses were performed at 50 °C, with H_2SO_4 0.01 N as eluent at a flow rate of 0.6 mL/min. Glucose standards (analytical reagent grade, Fischer Chemical) were used at concentrations in the range of 0.01–1.0 g/L. Styrene quantification was carried out by UV spectroscopy, as described by Mustarichie et al. (2018), using a Camspec M509T UV spectrometer. The cell-free supernatant was diluted in deionized water and the absorbance was measured at 246 nm. Styrene standards (synthesis grade, Sigma-Aldrich) were used at concentrations in the range of 0.005–0.1 g/L.

BC characterization

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was executed with a Perkin-Elmer Spectrum two spectrometer. The dried polymer samples were directly analyzed on the FTIR cells. The spectra were recorded between 400 and 4000 cm^{-1} resolutions with 10 scans, at 20 °C**.**

Scanning electron microscopy (SEM)

To evaluate the nanostructure of BC, the lyophilized samples were mounted for SEM observation using double-sided carbon tape and aluminium stubs and sputter-coated with a thin layer of iridium (150 T ES, Quorum, UK). The analysis was performed in a scanning electron microscope (Hitachi, Regulus 8220) using an acceleration voltage of 3 kV. The obtained SEM images were processed by public domain imaging software ImageJ (NIH image).

X-ray diffraction

The structural analysis of the dried samples was carried out by X-ray diffraction (XRD) using an X-ray diffractometer (PANalytical X′Pert PRO MRD) with a Cu Kα radiation source (45 kV and 40 mA) and wavelength of 1.540598 Å and equipped with an 1D X'Celerator detector. The XRD measurements were performed in the range of 10° to 65° (2θ) in the

Bragg–Brentano configuration, with a scanning step size of 0.033° in 2θ. Samples were mounted on a Si-0 background holder. The crystallinity index (CI) was estimated by the XRD deconvolution method as described by Park et al. (2010) using the X'Pert HighScore Plus software (Malvern Panalytical). Briefly, the crystalline and amorphous contributions were extracted by a Peak profile fit process using the diffraction intensity profiles. The analysis was restrained in the 2Theta range between 10° and 32°. Each peak was assumed a pseudo-Voigt function including a very broad peak near 21° assigned to the amorphous contribution. Furthermore, the background counts of the Si-0 background holder was also included in the analysis.

Thermogravimetric analysis

Thermogravimetric (TG) measurements were performed with a Simultaneous Thermal Analyser STA 449 F3 Jupiter from NETZSCH Thermal analysis (Wittelsbacherstraße, Germany), under a nitrogen atmosphere and loading 5 mg of each material into a covered aluminium crucible. The polymers were heated up to 500 °C at 10 K/min. The thermal degradation temperature (T_{deg}, °C) corresponds to the temperature value obtained for the maximum decreasing peak of the sample mass.

Mechanical properties

Puncture tests were performed at ambient temperature (22 °C) using a texture analyzer (Food Technology Corporation, Kent, UK), equipped with a 10 N load cell. The wet BC pellicles were fixed on a film support rig and submitted to perforation by a needle moving at 10 mm/s until rupture. The force at perforation (N) was calculated as the maximum puncture force until the breaking point. The deformation at perforation (mm) was determined as the maximum distance of deformation at the breaking point. Five replicas were analyzed for each membrane.

Permeability to O_2 and CO_2

The pure gas permeability of the BC membranes for O_2 and CO_2 was determined as described by Neves et al. (2010). For the measurements, a stainless-steel cell rig with two identical compartments separated by the supported BC membrane with an effective membrane area of 1 cm^2 was employed. Each gas permeability was assessed by pressurizing both compartments (feed and permeate) with a single gas and opening the permeate outlet until a driving force of ± 0.7 bar between compartments was established. The pressure change in both compartments was observed over time using two pressure transducers (Druck, PDCR 910 models 99,166 and 991,675, England). All assays were performed at a constant temperature, of 30 ºC, using a thermostatic bath (Julabo, Model EH, Germany).

Results and discussion

BC production

BC production by *K. xylinus* DSM 2325, DSM 2004, and DSM 46604 assays were conducted in styrene-supplemented HS medium, in which styrene was present at a low concentration (0.5 g/L) due to its low water solubility (Yeh et al. 2022), acting as a co-substrate. Higher concentrations of styrene resulted in a heterogeneous medium due to phase separation and/or were inhibitory for cell growth. Experiments with HS medium devoid of glucose were conducted to account for any BC production from the peptone and yeast extract available in the medium. Styrene was also tested as the sole carbon source. As shown in Table 1, all strains were able to grow in HS medium supplemented with styrene, although different BC production was observed.

K. xylinus DSM 2325 showed the highest BC production (2.70 ± 0.40 g/L) from the styrene/glucose mixtures (Table 1), with a product yield on a substrate basis of 0.25 g_{BC}/g_S. These values are higher

Table 1 Bacterial cellulose production by *K. xylinus* DSM 2235, DSM 2004, and DSM 46604 cultivated on HS medium supplemented with styrene and/or glucose

Carbon source (g/L)		BC (g/L)		
Glucose	Styrene	DSM 2325	DSM 2004	DSM 46604
–	–	–	0.29 ± 0.02	–
20	–	2.37 ± 0.26	2.05 ± 0.17	0.53 ± 0.04
–	0.5	–	0.32 ± 0.02	–
20	0.5	2.70 ± 0.40	1.57 ± 0.06	0.56 ± 0.06

than those found for the same strain grown with only glucose (2.37 ± 0.26 g/L and 0.24 g_{BC}/g_S, respectively), which demonstrates the utilization of styrene as a co-substrate to favor BC synthesis. These values fall within the range of those reported for other *K. xylinus* strains cultivated on glucose as the sole carbon source (0.24–6.23 g/L) (Yang et al. 2016; Andriani et al. 2020; Nascimento et al. 2021; Ogrizek et al. 2021). Other co-substrates have been successfully employed to enhance BC production, e.g. addition of 1% ethanol to the basal medium increased cellulose production by *K. hansenii* by 1.7 times (Park et al. 2003). Also, Ha et al. (2011) reported that medium supplementation with 1% glucuronic acid resulted in increased BC production by *K. hansenii* (from 7.4 to 10.5 g/L), as well as improvement in fiber diameter, along with higher tensile strength and water absorption capacity. *K. xylinus* PTCC 1734 also had improved BC production upon medium supplementation with low-quality date syrup (Moosavi-Nasab and Yousefi 2011). Therefore, despite *K. xylinus* DSM 2325 inability to use styrene as a sole carbon source, it was able to utilize it as a co-substrate with improved BC production.

Concerning *K. xylinus* DSM 46604, it presented the lowest BC production among all three tested strains, with a production of 0.53 ± 0.04 g/L (Table 1) on glucose, although among the range described in the literature (0.24–6.23 g/L) (Cavka et al. 2013; Yang et al. 2016; Ogrizek et al. 2021). Upon medium supplementation with styrene, BC synthesis was comparable to when using glucose as a sole carbon source 0.56 ± 0.06 g/L (Table 1). Similarly, to *K. xylinus* DSM 2325, *K. xylinus* DSM 46604 also did not yield any BC production from styrene as the sole carbon source. *K. xylinus* DSM 46604 was reported to synthesize BC from terephthalic acid, a plastic monomer from PET, both as the sole carbon source and as a co-substrate, reaching a BC production of 0.16 g/L and 0.70 g/L, respectively (Esmail et al. 2022).

BC production by *K. xylinus* DSM 2004 in styrene/glucose mixture assay (1.57 ± 0.06 g/L) was lower than in the glucose assay (2.05 ± 0.17 g/L) (Table 1), thus suggesting that the presence of styrene was detrimental to BC synthesis. Nonetheless, it was the only strain able to produce BC from styrene as the sole carbon source (0.32 ± 0.02 g/L), slightly higher than the production attained in the non-supplemented HS medium (0.29 ± 0.02 g/L) (Table 1). Cultivation on styrene as the sole carbon source also provided the highest product yield on a substrate basis (0.64 g_{BC}/g_S) when compared to glucose (0.20 g_{BC}/g_S), as well as the styrene/glucose mixture (0.15 g_{BC}/g_S). This bacterial strain has been previously reported to be able to use other synthetic plastics' monomers, such as, for example, terephthalic acid and ethylene glycol, as sole carbon sources, reaching BC productions of 0.81 g/L and 0.64 g/L, respectively (Esmail et al. 2022). Production of BC using aromatic compounds, namely naphthalene, has also previously been reported for *Starkeya* sp. strain N1B (Marín et al. 2019).

Overall, the obtained results show that all strains were able to synthesize BC upon cultivation in styrene supplemented media, with *K. xylinus* DSM 2325 and DSM 46604 achieving higher BC production. Interestingly, although with a low production, *K. xylinus* DSM 2004 was able to utilize styrene as the sole substrate.

BC characterization

Morphology of BC pellicles

The produced BC pellicles exhibited an orange coloration, except those synthesized in the media devoid of glucose, namely, in the non-supplemented HS medium and in the experiment run with styrene as the sole carbon source, in which they displayed a light-yellow tint (Fig. 1). After the alkaline treatment, all pellicles became translucent and displayed minor shrinkage, indicative of purification and removal of bacterial cell debris and medium remnants (Fig. 1).

The membranes' thickness was highly impacted by the cultivation conditions, besides also varying with the producing strain (Table 2). The thicker membranes were those produced by *K. xylinus* DSM 46604 from glucose as the sole carbon source (44 ± 2.2 µm) and in the glucose/styrene mixtures (22 ± 1.1 µm). These values are among those reported for BC membranes (20–200 µm) (Peres et al. 2016; Lee et al. 2017; Vázquez et al. 2021). However, considerably lower thickness values were observed for *K. xylinus* DSM 2325 and *K. xylinus* DSM 2004 BC produced in the glucose/styrene mixtures (6 ± 0.3 and 7 ± 0.4 µm, respectively), while even lower values were displayed by the BC membranes produced by *K. xylinus* DSM 2004 grown on styrene as sole carbon

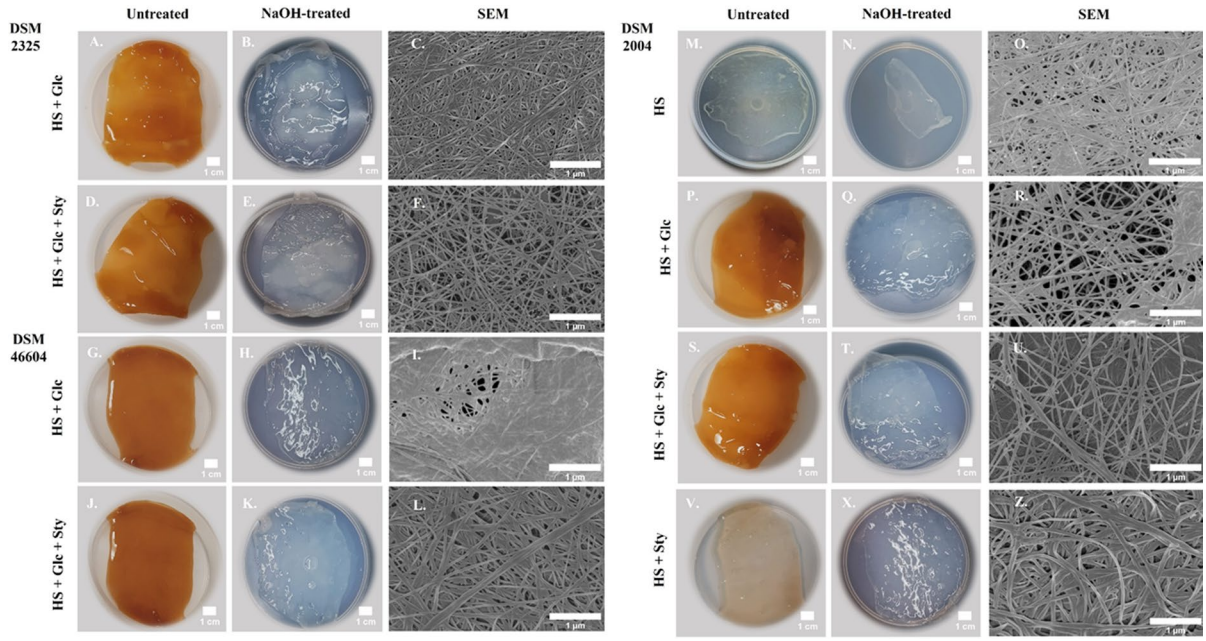

Fig. 1 Macroscopic and SEM images of BC pellicles produced by cultivation of *K. xylinus* strains DSM 2325, DSM 2004, and DSM 46604 on HS medium supplemented with glucose and/or styrene

Table 2 Characterization of the BC produced in the glucose and/or styrene supplemented assays by *K. xylinus* DSM 2325, DSM 2004, and DSM 46604 (CI, crystallinity index; degradation temperature, T_{deg}; n.a., data not available)

K. xylinus strain	Medium	Thickness (μm)	Fiber diameter (nm)	CI (%)	T_{deg} (°C)	Force at perforation (N)	Deformation at perforation (mm)	O_2 permeability (Barrer)	CO_2 permeability (Barrer)
DSM 2325	HS + Glc	20 ± 1.0	31 ± 5	41	329	0.19 ± 0.08	31 ± 3	10.2 ± 0.5	10.2 ± 0.5
	HS + Glc + Sty	6 ± 0.3	33 ± 4	46	324	0.27 ± 0.13	23 ± 3	1.1 ± 0.1	5.8 ± 0.3
DSM 2004	HS	2 ± 0.1	47 ± 13	41	337	0.09 ± 0.02	25 ± 4	0.1 ± 0.01	0.1 ± 0.01
	HS + Glc	12 ± 0.6	47 ± 11	34	327	0.03 ± 0.003	28 ± 2	62.2 ± 3.1	227.7 ± 11.4
	HS + Glc + Sty	7 ± 0.4	36 ± 6	36	318	0.05 ± 0.01	26 ± 7	2.5 ± 0.1	2.5 ± 0.1
	HS + Sty	3 ± 0.2	29 ± 4	33	327	0.04 ± 0.01	23 ± 4	1.8 ± 0.1	n.a
DSM 46604	HS + Glc	44 ± 2.2	37 ± 11	44	328	0.07 ± 0.03	34 ± 4	3.4 ± 0.2	3.4 ± 0.2
	HS + Glc + Sty	22 ± 1.1	44 ± 9	19	327	0.05 ± 0.004	28 ± 5	2.5 ± 0.1	42.3 ± 2.1

source (3 ± 0.2 μm) (Table 2), which is concomitant with the lower BC production observed in these conditions.

The SEM images (Fig. 1) revealed a nanostructure in line with that reported for BC membranes, specifically a porous structure with a three-dimensional network of ultrafine fibers on the nanometric scale (Kadier et al. 2021; Kim et al. 2021). Although some differences were observed for the BC synthesized by each strain and in the different tested media, fiber diameter varied from 29 to 47 nm for all samples (Table 2), which is in line with values reported for BC fiber networks (20–100 nm) (Choi and Shin 2020; Zhong 2020; Janeni and Adassooriya 2021).

The BC membranes produced by *K. xylinus* DSM 2325 were characterized by thinner fibers, with similar average diameters for the biopolymer synthesized from glucose (31 ± 5 nm) and from the styrene/

glucose mixtures (33 ± 4 nm). Despite the presence of styrene in the cultivation medium having no impact on the fiber diameter, the pellicles produced from glucose alone displayed a more compact nanostructure (Fig. 1c) than those synthesized in the presence of styrene that were more porous (Fig. 1f). The same trend was observed for the BC produced by *K. xylinus* DSM 46604, for which the pellicles synthesized from glucose alone were composed of a denser nanostructure (Fig. 1i). Nevertheless, the fibers synthesized in the presence of styrene were characterized by a higher average diameter (44 ± 9 nm) (Table 2).

K. xylinus DSM 2004 produced BC fibers with 47 ± 13 nm and 47 ± 11 nm in the non-supplemented HS medium and the HS medium with glucose, respectively (Table 2). Additionally, the pellicles synthesized from glucose displayed a less densely packed network (Fig. 1r). However, in contrast to strains *K. xylinus* DSM 2325 and DSM 46604, a significant decrease in the average fiber diameter was observed for the BC produced by *K. xylinus* DSM 2004 in the presence of styrene (36 ± 6 nm in the styrene/glucose mixture, and 29 ± 4 nm in the medium containing only styrene) (Table 2). These values are within the range reported for BC produced by *K. xylinus* DSM 2004 using other synthetic plastic monomers as carbon sources, namely terephthalic acid (33–68 nm) and ethylene glycol (41–74 nm) (Esmail et al. 2022). Nonetheless, *Starkeya* sp. strain N1B was described to synthesize BC from naphthalene with higher fiber diameters (60 nm) (Marín et al. 2019).

These results show that each strain was affected differently by the presence of styrene in the cultivation medium, resulting in BC membranes with different morphology, namely, variable thickness, fiber diameter, and porosity.

Fourier-transform infrared spectroscopy

Although some differences can be observed, the FTIR spectra of the BC produced by all *K. xylinus* strains under the tested cultivation conditions show the characteristic bonds described for the biopolymer (Gea et al. 2011). In particular, the stretching of OH groups present in BC is evidenced by the peak at 3346 cm^{-1} (Choi et al. 2004), while at 2896 cm^{-1} there is a band exposing the asymmetric stretching for CH_2 (Oh et al. 2005). Bending of OCH and HCH was revealed by a peak at 1427 cm^{-1} (Szymańska-Chargot et al. 2011) and the C-H bonds are defined by peaks at 1360–1315 cm^{-1} (Gea et al. 2011). Additionally, C–O–C asymmetric stretching and CH deformation were detected at 1162 cm^{-1}, as well as stretching of C–C rings in polysaccharides, related to the peak at 1109 cm^{-1} (Movasaghi et al. 2008). The bands at 1652 and 1544 cm^{-1} indicates the presence of amide bonds which may correspond to proteins from the culture medium or residual bacterial biomass that were not completely removed during sample purification (Akintunde et al. 2022).

Regarding the BC produced by *K. xylinus* DSM 2004, the FTIR spectra were similar for all tested media (Fig. 2a), indicating that for this strain the carbon source had no significant impact on the biopolymer's chemical structure, except for a slight decrease in the intensity of the peaks appearing at 800 and 1300 cm^{-1}. Similarly, for strains *K. xylinus* DSM 2325 and DSM 46604 (Fig. 2b), the spectra of the synthesized BC also appear to not be significantly influenced by the choice of medium, despite a slight decrease in the peaks' intensity in the presence of styrene for *K. xylinus* DSM 2325. Also, for the BC produced by *K. xylinus* DSM 46604, there was a decreased intensity of the peaks at 1300 and 1800 cm^{-1} (corresponding to the bending of OCH and HCH). These differences in peak intensity can be correlated to microstructural changes that can also impact the crystalline content of the samples (Riaz and Ashraf 2015), which will be further discussed. Also, the amide bond peaks were more evident in the case of BC grown in only glucose for *K. xylinus* DSM 46604, suggesting a need for further purification.

Crystallinity degree

The diffractograms of the BC produced by each strain from the different substrates, as well as the crystallinity index (CI) values are presented in Fig. 3 and Table 2, respectively. Most samples show the main reflections of the Iα X-ray diffraction pattern of crystalline BC, particularly, three narrow humps of varying intensity positioned at $2\theta = 15$, 17, and 23°, linked to the (100), (010), and (110) crystal planes (Wada and Okano 2001).

Regarding CI (deconvolution work shown in supplementary information), the highest values were observed for the BC synthesized by *K. xylinus* DSM 2325 grown in medium supplemented with glucose

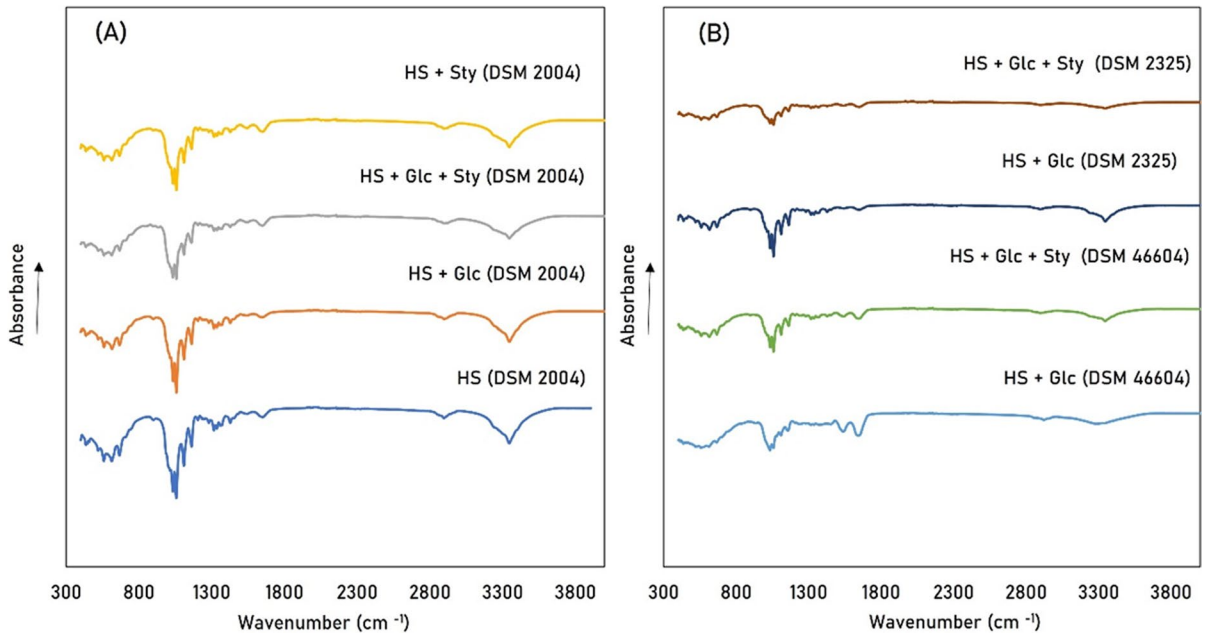

Fig. 2 FTIR spectra of the chemical groups present in BC grown in glucose and styrene mixtures by *K. xylinus* DSM 2004 (**a**) and DSM 2325 and DSM 46604 (**b**)

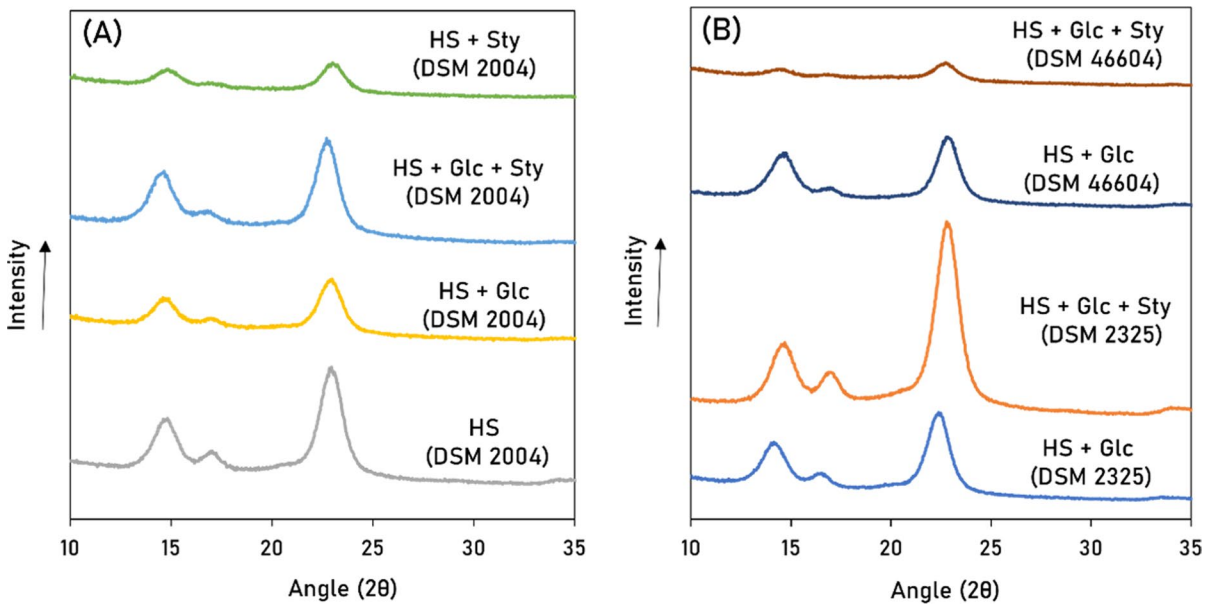

Fig. 3 XRD diffractograms of BC grown in glucose and styrene mixtures by *K. xylinus* DSM 2004 (**a**), DSM 2325 and DSM 46604 (**b**)

and styrene (46%) (Table 2), which is higher than the BC produced in the medium supplemented with only glucose (41%) (Table 2). For the BC produced by *K. xylinus* DSM 2004, the highest crystallinity was observed for non-supplemented medium (41%), followed by the BC cultivated in glucose and styrene mixtures (36%) while the mediums containing only glucose or styrene displayed the lowest CI (34 and 33%, respectively). *K. xylinus* DSM 2004 has been previously reported to produce BC with CI values of 57% and 40% from terephthalic acid and ethylene glycol as sole carbon sources, respectively (Esmail et al. 2022), which corroborates that different carbon sources impact polymer crystallinity. Concerning the BC synthetized by *K. xylinus* DSM 46604, it presented the lowest CI value overall, namely 19% when cultivated with a glucose and styrene mixture, compared to when only glucose was used for cultivation (44% (Table 2)). These results demonstrate that the CI differed depending on the producing strain and the cultivation medium. These variables have been described to influence CI, alongside the rate of BC synthesis, pH, and oxygen delivery (Pourramezan et al. 2009).

These results allow to compare the crystallinity of the samples and between samples. Similar analysis using the Segal methodology achieves much higher crystallinity Index values, but displaying the same evolution between samples. Although the Peak deconvolution methods may not be the more accurate to define cellulose crystallinity it takes into account an amorphous and a crystalline contribution and allows for a better description the observed diffraction patterns.

Thermogravimetric analysis

The thermograms of the produced BC pellicles (Fig. 4) show that all samples exhibited similar weight-loss behaviour, comprising three main degradation steps, which is concordant with what is reported for BC (Gayathri and Srinikethan 2019; Zhang et al. 2021). The first mass loss event (3–8%) occurred between 30–100 ºC and can be attributed to the loss of water from the samples (Zhang et al. 2021). The second and most prominent weight loss is relative to the main degradation of the polymer (Akintunde et al. 2022), and was observed between 225 and 375 ºC, with the lowest mass loss being for *K. xylinus* DSM 2325 BC produced from glucose (44%), and the highest for the biopolymer synthesized by *K. xylinus* DSM 2004 cultivated in the non-supplemented medium and in from styrene as the sole carbon source (67%). Between 380 and 500 ºC, the last degradation step was detected (weight loss of 7–14%) and can be assigned to the degradation of proteins or microorganisms that were not successfully removed during the purification procedure, as was evidenced by the amide bonds detected by FTIR analysis (Zhang et al. 2021). There was no significant variability among most of the samples' char yield (19–27%), except for the BC produced by *K. xylinus* DSM 2325 from glucose as the sole carbon source (40%).

The thermal degradation temperatures (T_{deg}) for the BC produced by *K. xylinus* DSM 2325 and DSM 46604 showed little disparity between the tested medium, presenting values of 329 and 328 °C for the biopolymer synthesized from glucose (Table 2), and of 324 and 327 °C for those produced from glucose/

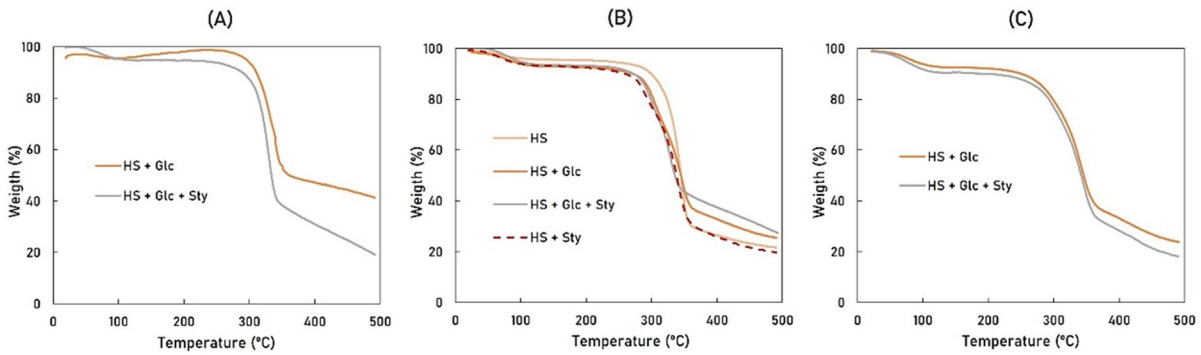

Fig. 4 TGA thermograms of BC produced by *K. xylinus* DSM 2325 (**a**), DSM 2004 (**b**), and DSM 46604 (**c**), in the different tested media

styrene mixtures, respectively (Table 2). On the other hand, *K. xylinus* DSM 2004 BC demonstrated a higher variation in values depending on the tested medium, showing degradation temperatures ranging from 318 °C (glucose/styrene medium) to 237 °C (non-supplemented HS medium) (Table 2). Moreover, the BC produced from glucose or styrene as sole carbon sources both presented an in-between value of 327 °C (Table 2). Nonetheless, the T_{deg} for all samples fall among the range reported for BC (310–399 °C) (Costa et al. 2017; Jia et al. 2017; Akintunde et al. 2022; Thongwai et al. 2022), including those synthesized from other aromatic compounds such as terephthalic acid (315–334 °C) (Esmail et al. 2022).

Mechanical properties

Puncture tests were performed to assess the produced BC membranes' mechanical properties. *K. xylinus* DSM 2325 wet BC membranes were clearly the ones with the highest resistance to perforation by the needle, with forces at perforation of 0.19 ± 0.08 N for the biopolymers produced from glucose, and of 0.27 ± 0.13 N (Table 2) for those produced from the styrene/glucose mixtures. On the other hand, the membranes differed in their flexibility, with the membrane produced from glucose presenting a higher perforation value (31 ± 3 mm) than that synthesized in the presence of styrene (23 ± 3 mm). These results indicate that the presence of styrene in the cultivation medium has apparently induced higher mechanical resistance to *K. xylinus* DSM 2325 BC membranes, concomitant with higher flexibility. The obtained values are lower than those reported for BC (0.6 N) produced by *K. xylinus* CECT using glucose as a carbon source (Cazón et al. 2019; Vázquez et al. 2021), which can probably be linked to a higher membrane thickness (20 µm) than in this study (6 µm). Interestingly, the deformation at perforation values obtained for *K. xylinus* DSM 2325 membranes are higher than that reported for BC (0.39 mm) (Cazón et al. 2019; Vázquez et al. 2021), as well as high-density polyethylene (HDPE) (16 mm) (Yang et al. 2012) or polypropylene (PP) (1.3 mm) (Yang et al. 2012) and close to the values described for PET (29 ± 8 mm) (Patti and Acierno 2020), revealing a high ability to be deformed before bursting.

The BC produced by *K. xylinus* DSM 46604 also displayed a higher deformation at perforation for the biopolymer produced from glucose (34 ± 4 mm) compared to that synthesized from the styrene/glucose mixture (28 ± 5 mm) (Table 2). However, *K. xylinus* DSM 46604 membranes had considerably lower mechanical resistance, as shown by their very low force at perforation values (0.07 ± 0.03 and 0.05 ± 0.004 N, respectively). Concerning *K. xylinus* DSM 2004 BC membranes, they also showed low forces at perforation, similar to DSM 46604 BC, varying from 0.03 ± 0.003 N to 0.09 ± 0.02 N (Table 2), with the highest value being attributed to the biopolymer produced in the non-supplemented medium and the lowest for the one produced from glucose. The corresponding deformation at perforation values were also similar and varied from 23 ± 4 N to 28 ± 2 N, mainly depending on the membrane's thickness, where higher deformation at perforation can be observed for higher membrane thickness. These results show that the BC synthesized by *K. xylinus* DSM 2004 and DSM 46604 were flexible but presented low mechanical resistance.

Permeability to O_2 and CO_2

The permeability of O_2 and CO_2 for the wet BC membranes was investigated to evaluate their barrier properties. In general, the membranes' permeability to CO_2 (0.1–227.7 barrer) was either similar to or higher than the permeability to O_2 (0.1–62.2 barrer) (Table 2). This is expected since the membranes were in their wet form and the permeability followed the same trend of each gas in water, where CO_2 has higher permeability than O_2 (1923 vs 91.0 barrer) (Lide 2008). Water molecules adsorb into the polymer matrix and may increase the fractional free volume of the membranes, playing a key role in enhanced gas transport (Tomé et al. 2010).

The BC membranes produced by *K. xylinus* DSM 2325 from the styrene/glucose mixtures showed lower O_2 and CO_2 permeability values (1.1 ± 0.1 and 5.8 ± 0.3 barrer, respectively) than the membranes synthesized from glucose (10.2 ± 0.5 and 10.3 ± 0.5 barrer, respectively) (Table 2). The same trend was observed for the BC produced by *K. xylinus* DSM 2004, though considerably higher values were attained for the membranes produced from glucose (62.2 ± 3.1 and 227.7 ± 11.4 barrer, respectively). These values differ from those reported by Tomé et al. (2010) for BC produced under similar

conditions by the same strain, namely, an O_2 permeability of 10 ± 0.5 barrer and a CO_2, permeability 187 ± 9.4 barrer. The higher permeability observed for both gases can be explained by the higher porosity of the matrix, which can be observed in Fig. 1.

For *K. xylinus* DSM 46604, the presence of styrene in the cultivation medium has apparently had little effect on the membranes in terms of O_2 permeability, as similar values were attained for both samples (3.4 ± 0.2 barrer for the BC produced from glucose and 2.5 ± 0.1 barrer for that produced from the styrene/glucose mixture). For CO_2, on the other hand, the permeability of the membranes was much higher for the BC produced in the presence of styrene (42.3 ± 2.1 barrer) compared to that synthesized from glucose (3.4 ± 0.2 barrer).

Overall, these results indicate that it is possible to tune BC membrane's permeability to CO_2 and O_2 by a selection of the appropriate strain and cultivation media, which is valuable when there are specific barrier property needs for different purposes such as packaging, where lower gas permeability is required (0.21–7.3 barrer for O_2 and 1.2–90 barrer for CO_2 (Bao et al. 2006; Semsarzadeh et al. 2008; Paunonen 2013; Michiels et al. 2017)), or biomedical applications, where higher gas permeability is essential (8.4–140 barrer for O_2 and 172–950 barrer for CO_2 (Roy et al. 2010; Eusébio et al. 2020; Tran et al. 2020)).

Overall assessment of the produced BC membranes properties

The BC membranes produced in this study showed differing properties depending on the producing strain and the culture medium. The BC produced *by K. xylinus* DSM 2325 grown in HS medium supplemented with glucose and styrene presented the highest crystallinity among all strains and media (CI = 46%), average gas permeability (10.2 ± 0.5 barrer for O_2 and 10.3 ± 0.5 barrer for CO_2) and force at perforation (0.19 ± 0.08N). Moreover, when the medium was co-supplemented with styrene, an increased resistance at perforation (0.27 ± 0.13 N) was observed, together with lower permeability to both O_2 (1.1 barrer) and CO_2 (5.8 barrer). These features render these produced membranes of interest for the development of packaging or coatings applications. *K. xylinus* DSM 2004 cultivated in a non-supplemented HS medium provided a more crystalline polymer in comparison to other media (CI = 41%) with low permeability (0.1 ± 0.01 barrer) to both O_2 and CO_2, as well as a higher force at perforation among all membranes from this strain (0.09 ± 0.02 N), making it fit for packaging purposes. Still, the biopolymer produced from glucose had a lower crystallinity (CI = 34%) but its permeability increased to 62.2 ± 3.1 barrer for O_2 and to 227.7 ± 11.4 barrer for CO_2, rendering such membranes suitable for biomedical applications. For products that require a thicker membrane (44 µm), with lower crystallinity (CI = 19%), the BC produced by *K. xylinus* DSM 46604 from HS medium with glucose and styrene is a promising option.

Conclusions

Komagataeibacter xylinus strains DSM 2325, DSM 2004, and DSM 46604 produced BC in styrene containing media. All strains grew on glucose/styrene mixtures, but only *K. xylinus* DSM 2004 was able to utilize styrene as the sole carbon source. While the BC produced in all tested media had comparable physico-chemical properties in terms of structure and thermal degradation behaviour, the produced biopolymers differed in their microstructure, mechanical properties and permeability to gases. Therefore, this study opens up the possibility of producing BC with varying properties according to specific applications needs, while contributing to reduce the burden of plastic pollution by using styrene as a co-substrate in the medium.

Acknowledgments The authors acknowledge the National Natural Science Foundation of China (Grant Numbers: Institute of Microbiology, Chinese Academy of Sciences: 31961133016; Beijing Institute of Technology: 31961133015; Shandong University: 31961133014) for support of the Project Bio Innovation of a Circular Economy for Plastics (BioICEP).

Authors' contributions Conceptualization: AE, CAVT and FF; Methodology: AE, POA, ACM, JVP and AG; Formal analysis and investigation: AE, JVP and LAN; Writing—original draft preparation: AE; Writing—review and editing: CAVT, LAN, MAMR and FF; Funding acquisition: MAMR and FF; Resources: LAN, MAMR and FF; Supervision: CAVT and FF.

Funding This work was financed by national funds from FCT/MCTES—Fundação para a Ciência e a Tecnologia, I.P., Ministério da Ciência, Tecnologia e Ensino Superior, in the scope of the projects UIDP/04378/2020 and UIDB/04378/2020

of the Research Unit on Applied Molecular Biosciences—UCIBIO, project LA/P/0140/202019 of the Associate Laboratory Institute for Health and Bioeconomy—i4HB, projects UIDB/50006/2020 and UIDP/50006/2020 of the Associate Laboratory for Green Chemistry—LAQV, and projects LA/P/0037/2020, UIDP/50025/2020 and UIDB/50025/2020 of the Associate Laboratory Institute of Nanostructures, Nanomodelling and Nanofabrication—i3N, and by the European Union's Horizon 2020 research and innovation programme through Project Bio Innovation of a Circular Economy for Plastics (BioICEP), under grant agreement No 870292. Asiyah Esmail and Paloma Ortiz-Albo acknowledge FCT I.P. for PhD Grants 2021.05014.BD and SFRH/BD/139389/2018, respectively.

Availability of data and materials Data and materials will be provided on request.

Declarations

Conflict of interests The authors declare no competing interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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