Microbial cellulose from a Komagataeibacter intermedius strain isolated from commercial wine vinegar

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ABSTRACT

In this study a new bacterial producer isolated from commercial vinegar is identified as *Komagataeibacter intermedius* JF2 based on the examination of general taxonomical characteristics, 16S rDNA sequence analysis, and MALDI-TOF mass spectrometry. The membrane of cellulose produced is studied in terms of morphology by scanning electron microscope, crystallinity by X-Ray Diffraction, structure by Fourier transform infrared spectroscopy, and water absorption capacity. Bacterial cellulose yield and characteristics of the membrane produced by the new isolated JF2 are compared with those of the well-known and commonly-used bacterial cellulose producer *Komagataeibacter xylinus*. Yield of cellulose production was higher for JF2 than for *K. xylinus* grown on several culture media. The nanocellulose fibers produced by JF2 showed a higher degree of crystallinity and a more homogeneous size distribution than those of *K. xylinus*. The results suggest that *Komagataeibacter intermedius* JF2 could contribute to better meet the requirements for new biotechnological applications of the bacterial cellulose.

INTRODUCTION

Cellulose is an organic polymer composed of β-1,4-linked D-glucose used in the elaboration of numerous industrial products such as paper, textiles, food additives and pharmaceutical devices. Moreover, materials science has demonstrated increasing interest in cellulose because of its great potential as a reinforcement material in composites owing to its mechanical and physical

properties.[1] Cellulose is very abundant in nature as part of the cell wall of vegetal cells, and plants have traditionally been the main source of this material. However, plant-derived cellulose needs to be purified of hemicelluloses and lignin by enzymatic, chemical and/or mechanical treatments before further used.[2,3] These processes have a high economic and environmental cost and, in addition, could change the functionality of the cellulose and limit its applicability.[3] Some bacteria are able to synthesize cellulose, including the genera *Agrobacterium,*[4,5] *Rhizobium*[6] and *Pseudomonas,*[7] and specially members of the *Acetobacteriaceae* family as *Komagataeibacter.*[8,9] *Komagataeibacter xylinus* (previously known as *Gluconacetobacter xylinus*[10] or *Acetobacter xylinus*,[8] is the most studied species and one of the few with substantial cellulose-production yields to be commercially exploited.[11]

Bacterial cellulose (BC) is a linear extracellular polysaccharide with the same chemical composition of plant cellulose, but its conformation and physicochemical properties are different, conferring it superior qualities. BC exhibits higher chemical purity, because it is produced free of hemicellulose and lignin, higher degree of crystallinity and higher water retention capacity.[12,13] Moreover, BC presents great elasticity, high tensile strength and good biocompatibility. These unique properties make BC a multifunctional biomaterial and enable many successful applications, especially in the areas of biomedical, catalysis, conducting materials and electric devices.[12] BC has been proposed for strength reinforcement of polymeric materials or paper [14,15], as a thickening agent and food stabilizer,[16] for food packaging,[17,18] as a biomaterial for manufacturing cosmetics,[19] as artificial skin,[20,21] for artificial blood vessels or tissue engineering;[22–24] as diaphragms for loudspeakers[25,26] and for the preparation of optically transparent films,[27] electric conductors[28] or magnetic materials.[29,30]

BC production can be achieved by culturing the producer bacterium on a glucose-rich liquid medium, in static conditions. Glucose is polymerized and extruded outside the cell by the activity of the membrane-associated cellulose synthase complex. Individual cellulose chains are tied by hydrogen bonds into subelementary fibrils that assemble with adjoining subelementary fibrils giving rise to microfibrils that gather into 20 – 70 nm wide ribbons.[31] The result is a tri-dimensional network of cellulose nanofibrils that builds up at the air-liquid interface in the form of a dense pellicle or membrane that, after several days of incubation to allow appropriate thickness, can be collected. Physical and mechanical properties of pellicle could be strongly influenced by culture conditions, carbon source and, more importantly, by the bacterial strain that synthetize the cellulose.[32,33] Properties such as degree of crystallinity, water content capacity or tensile strength, could determine the applicability of bacterial cellulose, especially in those cases in which bacterial cellulose is used as a matrix to obtain composites.[34,35] On the other hand, an issue that restrains commercial production and extended application of BC is the low yield of the described BC-producing strains.[36] The isolation of new strains from the natural habitats of BC-producing bacteria as fruits, beverages and vinegar is a strategy often used in order to obtain strains with higher efficiency.[37–40] Thus, the isolation of strains that produce bacterial cellulose with improved characteristics and with substantial production yields is desirable both, to extend the applicability of bacterial cellulose and to improve its commercial exploitation.

In this study, some bacterial cellulose producer strains were isolated from wine vinegar. The strain JF2 showed the highest production yield and was identified according to molecular and biochemical characteristics as a member of *Komagataeibacter intermedius*. Its capability of producing cellulose in different culturing conditions was investigated and compared with that of the reference strain *Komagataeibacter xylinus*. The properties of the cellulose pellicles of both, new isolate and

reference strain, were analyzed in terms of their chemical structure, crystallinity, water absorption capacity, and SEM morphology. The results indicated that *Komagataeibacter intermedius* JF2 produces cellulose with higher efficiency and higher crystallinity.

EXPERIMENTAL

Microorganism isolation and culture conditions

The isolation of cellulose producing bacteria was carried out from commercial wine vinegar, using the Hestrin-Schramm (HS) medium (20 gL^{-1} glucose, 5 gL^{-1} peptone, 5 gL^{-1} yeast extract, 2.7 gL^{-1} Na_2HPO_4 and 1.15 gL^{-1} citric acid, pH 6).[41] Two mL of sample wine vinegar were inoculated in 20 mL of liquid HS culture medium. After 5 days of incubation at 28 °C in static condition, tubs that presented BC pellicles on the surface of the culture were selected. From those, 0.1 mL samples were spread in HS-agar plates and incubated at 28 °C to obtain single colonies. Pure cultures of each grown colony were obtained by streaking repeatedly onto HS-agar plates. Isolates were individually tested for BC production by inoculation in test tubes containing 10 mL of HS liquid medium and incubating at 28 °C for 8 days. Cellulose production was detected by the appearance of a pellicle on the air/liquid interface of culture broth. The bacterial cellulose producer isolated strains were preserved under freezing at -80°C in a Revco deep freezer, using 20% glycerol cell cryoprotectant.

To assess BC productivity on different carbon sources, HS medium was modified as follows: *HS-glucose-mannitol* containing HS based medium (5 gL^{-1} peptone, 5 gL^{-1} yeast extract, 2.7 gL^{-1} Na_2HPO_4 and 1.15 gL^{-1} citric acid), 10 gL^{-1} glucose and 20 gL^{-1} mannitol; *HS-mannitol* containing HS based medium and 20 gL^{-1} mannitol; *HS-ethanol* containing HS based medium and 5 % (v/v) ethanol.

Komagataeibacter xylinus 7351 T from the Spanish Type Culture Collection (CECT) was used as the reference strain.

Bacterial cellulose production

Culture inoculum to produce bacterial cellulose was prepared by transferring bacterial cells grown on HS–agar to HS liquid medium. After shaking vigorously the resulting cell suspension of an $OD_{600\,nm}$ of 0.6 was used to inoculate (1:40) 10 cm–Petri dishes containing 40 mL of HS or modified HS media. All the BC production experiments were conducted at least three times. The cultures were statically incubated at 28 °C for 7 days. For time course BC production, incubation was interrupted at 24 h periods. After incubation, bacterial cellulose pellicles generated in the air/liquid interface of the culture medium were harvested, rinsed with water and incubated in 1% NaOH at 70 °C overnight to eliminate bacterial cells. Finally, the BC pellicles were thoroughly washed in deionized water until the pH reached neutrality. Purified cellulose was dried at 40 °C until constant weight was reached. To evaluate the production yield, the amount in grams of dried BC per litre of the original culture medium was determined (gL^{-1}). Dry membranes were used to determinate morphological properties and to perform XDR and FT-IR analysis.

Identification of bacterial cellulose producing strain

Molecular identification

The isolated strains were identified by 16s rDNA sequencing. Genomic DNA was extracted from pure culture pellet with GeneJET Genomic DNA Purification Kit (Thermo Fischer) kit. Universal primers for Bacteria domain, 27F (5´-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3'), were used for the amplification of a partial 16s rDNA gene region by the polymerase chain reaction (PCR) using PCR machine (GeneAmp PCR System 2400). The PCR mixture volume was 50 µL containing 25 µL mix (buffer, $MgCl_2$, dNTPs, ddNTPs and polymerase), 1 µL each primer, and 1 µL DNA template. The partial 16s rDNA region was sequenced by Sanger (Centres Científics i Tecnològics, UB). Sequences were compared with those from the GenBank database using BLAST. MALDI-TOF MS-Protein mass fingerprints (MALDI Biotyper Systems) was used to identify JF2 strain. Partial 16S rDNA sequence from JF2 strain was deposited in the GenBank database

under the accession number SUB4239423 seq1 MH553300. Amplification of the *bcsA* gen was performed with primers CELS3_Fw and CELS3a_Rv as described previously.[42]

Physiological characteristics

Initial phenotypic characterization was carried out according to Bergey's Manual of Systematic Bacteriology.[43] Colonies grown on HS medium at 28 °C for five days were used to observe cell morphology, Gram-staining reaction and to perform physiological and biochemical analyses. Presence of catalase, oxidase reaction, production of acetic acid, growth on sole carbon sources as D-glucose, D-mannitol, sucrose, D-arabinose, D-rafinose and ethanol; requirement of acetic acid for growth was evaluated. Capability of cellulose production on D-glucose, D-mannitol, D-glucose + D-mannitol, ethanol and D-glucose + ethanol at different concentrations and pH was tested.

Characterization of bacterial cellulose pellicles

Water holding capacity (WHC)

Dried BC pellicles were weighted and immersed in deionized water for 24 h. After 24 h excess of water was removed and the weight was measured. The WHC was expressed as:

$$\mathrm{WHC} = \frac{\mathrm{W_{wet-}W_{dry}}}{\mathrm{W_{dry}}} \quad (1)$$

where W_{wet} is the weight of wet pellicle and W_{dry} is the initial weight of the dried pellicle.

Scanning electron microscope (SEM)

Dried BC pellicles were analyzed by SEM (JSM 7100 F) using a LED filter. Average diameter of BC microfibrils was obtained using ImageJ software.

X-Ray Diffractometry (XRD)

Dried BC pellicles were subjected to X-ray diffractometry analysis (PANalytical X'Pert PRO MPD Alpha1 powder diffractometer). The samples were analyzed at the radiation wavelength of 1.5406 Å. Samples were scanned from 2 to 50°, 2Θ range. Samples were fixed over a zero background Silicon single crystal sample holder (pw1817/32), and the ensembles were mounted in a PW1813/32 sample holder. All the replicates of each sample were measured with the same Silicon holder. The crystallinity index (CI) of produced bacterial cellulose was calculated based on equation (2):[44]

$$\mathrm{CrI}(\%) = \frac{\mathrm{I_c} - \mathrm{I_{am}}}{\mathrm{I_c}} \times 100 \quad (2)$$

where I_c is the maximum intensity of the lattice diffraction and I_{am} is the intensity of the peak at 2Θ = 18°, which corresponds to the amorphous part of cellulose. The intensity of the peaks was measured as the maximum value obtained for the peak taking into account a baseline.

Fourier transform infrared spectroscopy (FT-IR)

FTIR spectra of BC pellicles were recorded in duplicate at room temperature using an ATR-FTIR spectrophotometer (Spectrum 100, Perkin Elmer, USA). FTIR spectral analyses were conducted within the wavenumber range of 600-400 cm^{-1}. A total of 64 scans were run to collect each spectrum at a 1 cm^{-1} resolution.

RESULTS AND DISCUSSION

Isolation and identification of BC-producing bacteria

Six different brands of commercially available wine vinegar were screened for cellulose producing bacteria. Prior the isolation, 2 mL of vinegar samples were enriched with 20 mL of HS medium. This step was found necessary to obtain isolates from vinegar, an extreme medium with limited culture recovery of

microorganisms.[45] After several days of incubation at 28 °C, culture tubs that presented bacterial cellulose pellicles were used to obtain single colonies by spreading 0.1 mL samples in HS-agar plates. After 5 days of incubation, several distinct colonies growing randomly on the surface of the agar were observed. Twenty single isolates were selected according to distinctive colony morphology. The isolates were obtained in pure culture after streaking on HS-agar. Subsequently, strains were inoculated in HS liquid medium to evaluate their capacity to produce cellulose. After 8 days of incubation, four strains were able to synthesize cellulose as a membrane at the air/liquid interface of culture test tubes. Interestingly, all four BC-producing strains were isolated from the same brand of vinegar, distinguished by making wine vinegar by the traditional method, which could preserved the natural population of bacteria.

BC production efficiency of the isolated strains was determined and compared with that of the reference strain *K. xylinus*. Production capabilities were in the range of 0.4 – 1.2 gL^{-1}. As shown in Figure 1, the strains JF1, JF2 and JF4 presented higher BC yield than the reference strain *K. xylinus*. These results are comparable with those obtained with common cellulose producing isolates growing in glucose-rich media.[46,47] The strain JF2 presented the highest BC production yield (1.2 gL^{-1}), 48 % higher than *K. xylinus*. Thus, JF2 was selected for identification and further characterization.

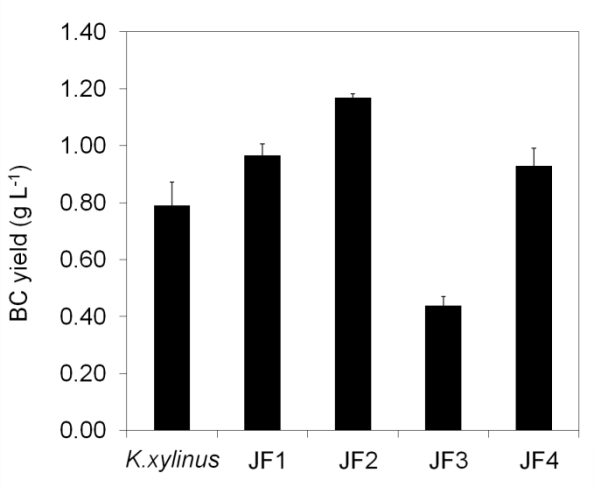

Figure 1. Bacterial cellulose production of the isolated strains and *K. xylinus* grown on HS medium.

Isolated BC-producer JF2 was presumably identified by submitting the corresponding 16S rDNA sequence to BLAST analysis. Results showed that JF2 belonged to the genera *Komagataeibacter* (Table 1). *Komagataeibacter* is a member of the acetic acid bacteria group frequently found in fruits and vinegar, and includes numerous cellulose producing species.[48] However, bacteria from this group share high degree of homology, and molecular identification based on 16S rDNA sequences fails to discriminate between species closely related. Siever and Swings (2005) reported that *Gluconacetobacter europaeus*, *G. xylinus*, *G. intermedius* and *G. oboediens* present more than 99% 16S rDNA gene sequence similarity.[49] MALDI-TOF MS fingerprinting technique has been shown to be useful to discriminate among species of genera *Komagataeibacter*.[50] Analysis by MALDI-TOF MS fingerprinting confirmed that JF2 strain belonged to *K. intermedius* (Figure 2). The new isolated was named *K. intermedius* JF2. Additionally, the presence of the *bcsA* gene in the genome of strain JF2, encoding the cellulose synthase, was demonstrated by its amplification with consensus primers. Results indicated that *bcsA* gene of JF2 shared 100 % identity with *bcsA* gene of type strain *Komagataeibacter intermedius* TF2 (Table 2).

Table 1. Molecular identification of the isolated strains from 16S rDNA analysis.

	Description	Ident	e-value
JF1	*G. europaeus*	99 %	0.0

	K. xylinus E25	99 %	0.0
JF2	*K. oboediens* JCM 16937	98 %	0.0
	K. intermedius JCM 16936	98 %	0.0
JF3	*G. europaeus* strain 3Pe4	99 %	0.0
	K. xylinus E25	99 %	0.0
JF4	*K. xylinus*	99 %	0.0
	G. europaeus KGMA0119	99 %	0.0

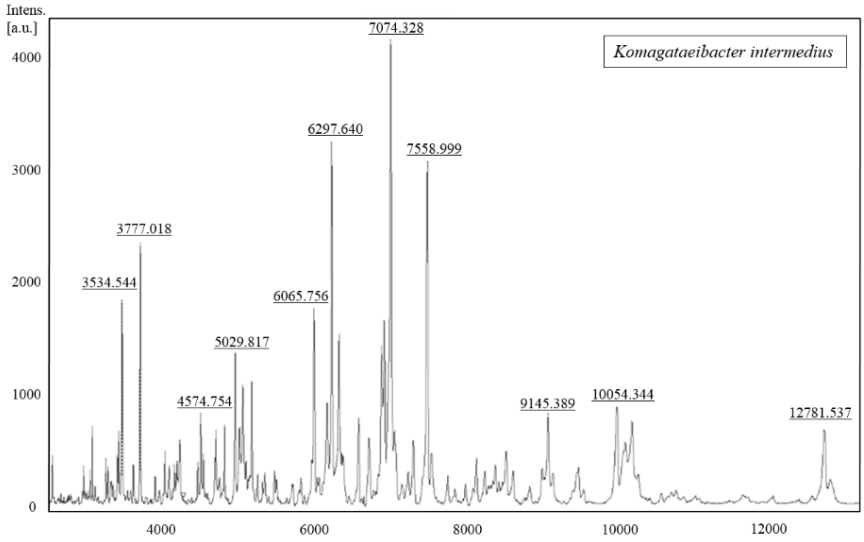

Figure 2. MALDI-TOF MS spectra from JF2 strain.

Table 2. Alignment results from BcsA protein sequence using BLAST.

	Description	**Ident**	**e-value**
JF2	*Cellulose synthase catalytic subunit AB [Komagataeibacter intermedius TF2]*	100 %	4e-36

Morphological and biochemical characterization of *K. intermedius* JF2 was carried out. JF2 cells were gram-negative rods, approximately 1.7 µm long and 0.45 µm wide. Biochemical and physiological traits for reference and isolated strain are shown in Table 3. The two microorganisms presented similar biochemical characteristics. Both were oxidase negative and catalase positive, and were able to grow on glucose, mannitol, sucrose and arabinose. However, JF2 was not able to grow on rafinose in contrast with *K. xylinus*. On the contrary, JF2 was able to grow with ethanol as sole carbon source, suggesting its capability to oxidize ethanol to acetic acid, as has been described for *K. intermedius* strains.[51] Regarding the sugars devote to cellulose production, JF2 was able to synthetize cellulose from glucose, mannitol and, at a less extend, from ethanol (Table 3).

Table 3. Physiological characteristics of strain JF2.

	***K. xylinus* 7351 T**	***K. intermedius* JF2**	***K. intermedius* description in Bergey's Manual**
Growth on carbon sources			
D-glucose	+	+	+
D-Mannitol	+	+	ND
Sucrose	+	+	+/-
D-Arabinose	+	+	ND
D-Rafinose	+	-	ND
Ethanol	-	+	+
Cellulose production on			
D-glucose	+	+	+
D-Mannitol	-	+	ND
D-Glucose + D-Mannitol	weak	+	ND
Ethanol 5 %	-	weak	weak
D-Glucose + Ethanol 5 %	-	+	ND
Acetic acid production	+	weak	ND
Requirement of acetic acid for growth	-	-	-

ND: no data

Bacterial cellulose production

As stated above, *K. intermedius* JF2 presented higher BC yield growing on HS medium than reference strain *K. xylinus*, showing an increment of 32.8 %. JF2 was able to grow on mannitol, as described for strains of

Komagataeibacter.[49] However the capability of *K. intermedius* to produce cellulose growing on mannitol has not been described so far. In this work, the effect of mannitol on the production of bacterial cellulose was investigated and compared with *K. xylinus*. Strains were grown in modified HS medium (HS-mannitol) in which D-glucose, the original carbon source, was replaced by mannitol. The effect of standard HS medium supplemented with mannitol (HS-glucose-mannitol) was determined as well. As shown in Figure 3, JF2 was able to produce cellulose growing in mannitol as sole carbon source. Its BC yield was higher than that of *K. xylinus* growing on glucose. The BC production was even higher when JF2 grew with HS-glucose-mannitol. *K. xylinus* grew on mannitol, but was unable to produce cellulose. For bacterial cellulose producers, carbon source is used to both, increase of biomass and cellulose synthesis. The among of carbon source that is devoted to one or other function is both carbon source and strain depending.[52–54] D-mannitol seemed to be a more suitable carbon source to produce cellulose than D-glucose for *K. intermedius* JF2. For this strain, D-mannitol is probably transformed to D-fructose and then metabolized to BC.[55]

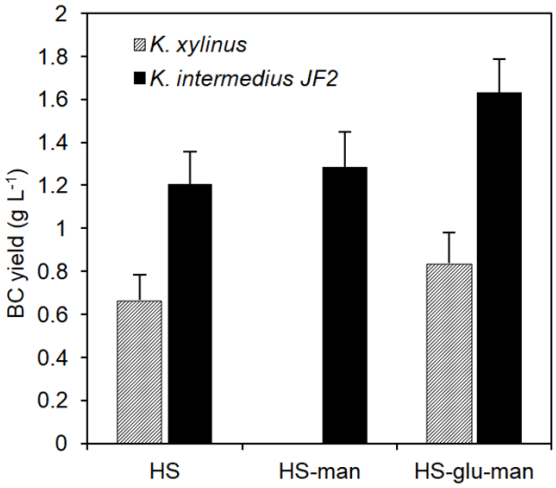

Figure 3. Bacterial cellulose production of *K. intermedius* JF2 and *K. xylinus* in HS, HS-mannitol (HS-man) and HS-glucose-mannitol (HS-glu-man).

To investigate the effect of pH on growth and cellulose production, the bacteria were cultured in HS broth at different starting pH (from 3 to 8.5) and incubated for 7 days at 28 °C. The pH was adjusted varying the relative concentration of Na_2PO_4/citric acid. After the incubation time, the final pH was measured and the presence

of cellulose was determined. *K. intermedius* JF2 and *K. xylinus* were able to produce cellulose on HS medium with a starting pH ranging from 3.5 to 7, being 5.5 – 6 the optimum starting pH. Growth was not detected at starting pH of 8 and 8.5. After 7 days of incubation a decrease on the pH of the culture was recorded. However, the acidification of the culture was less noticeable in *K. intermedius* JF2 for all the starting pH (results not shown). Figure 4 shows the time course profiles of pH values during bacterial growth and cellulose production for both strains growing on HS and HS-glu-man medium starting at optimum pH 5.5 - 6. A drop in pH was observed during the first three days of incubation of the strains. However, it should be noted that the culture of *K. xylinus* decreased the pH value from 6 to 3, while cultures of *K. intermedius* JF2 presented only a 1-point decrease in pH, both on HS and HS-glu-man media, corroborating the results obtained previously. The acidification of the culture medium can be attributed to several causes based on the metabolic activity of the strains during growth. It has been described that the activity of the enzyme glucose dehydrogenase located in the outer membrane of *K. xylinus* oxidizes glucose to gluconic acid extracellularly which decrease the pH of the medium.[56,57] For some strains of *Komagataeibacter*, more than 70% of the initial glucose can be derived to gluconic acid. [58] Eventually the acidity of the medium could hamper the bacterial growth and the biosynthesis of BC.[59] Among the strains belonging to *K. intermedius* the oxidation of glucose to gluconic acid is a variable trait.[51] Our results suggested that the higher conversion yield of glucose to BC of the isolate *K. intermedius* JF2 on comparison with *K. xylinus* could be explained by its capability to maintain the pH of the medium stable during growth.

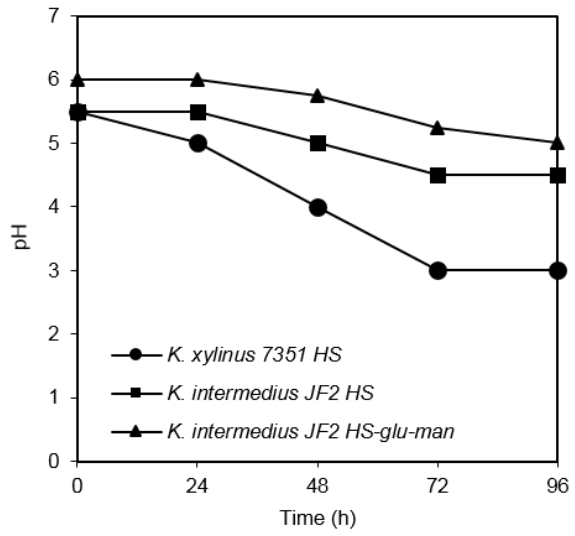

Figure 4. Time course profiles of pH values during *K. xylinus* and *K. intermedius* JF2 growing on HS and HS-glu-man.

The dynamics of BC production was evaluated for *K. xylinus* and *K. intermedius* JF2 growing on HS and HS-glu-man respectively, during a time course of 9 days. The amount of cellulose produced was recorded at 24 h intervals over the 9 days (Figure 5). The maximum cellulose yield was obtained after 6 days for *K. intermedius* JF2 and after 9 days for *K. xylinus*. Likewise, JF2 presented higher production efficiency than *K. xylinus*, corroborating the results previously obtained. The results suggested that strain JF2 has potential to be used as bacterial cellulose producer at large scale for commercial application, since it was able to accumulate more cellulose than *K. xylinus* in a shorter period of time.

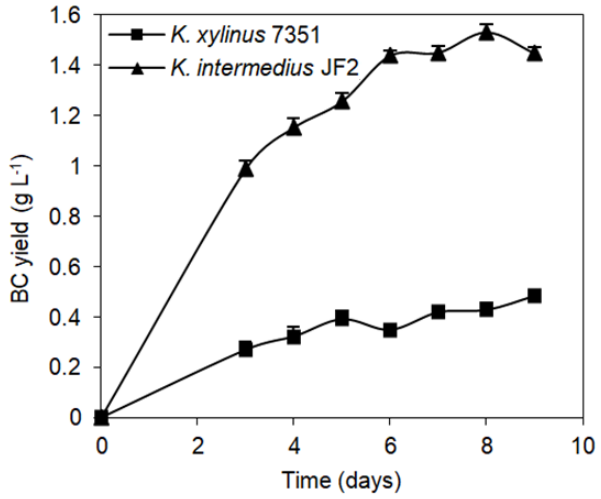

Figure 5. Profiles of pH values during growth of *K. xylinus* and *K. intermedius* JF2 in HS and HS-glu-man, respectively.

Bacterial cellulose pellicles characterization

BC membranes produced by *K. intermedius* JF2 and K. *xylinus* were characterized in terms of morphology, chemical structure and crystallinity by SEM, FTIR and XRD, respectively. Water absorption capacity of the BC membranes was also measured.

Scanning electron microscope (SEM) analysis

Porous structure and fibril distribution of the BC pellicles were analyzed by SEM (Figure 6). The pellicles from cultures of *K. intermedius* JF2 on HS medium exhibited a dense network of fibrils evenly distributed, similar to that of reference strain *K. xylinus*. Randomly measurements of 100 nanofibrils from SEM images analyzed by ImageJ software resulted on a fibril diameter size of 72 ± 12 nm and 58.2 ± 16 nm for *K. intermedius* JF2 and *K. xylinus* respectively. Thus, the two strains grown in the same culture medium and conditions generated cellulose fibers with different morphological characteristics. Nanofibers from JF2 were wider and showed less size dispersion than nanofibers from *K. xylinus* (Figure 6). The morphology and size distribution of the

nanofibers influence the 3D-structure of the matrix of cellulose, affecting porosity and density, which in turn could impact the physical and mechanical properties of the resulting membrane,[33] and in its applicability.

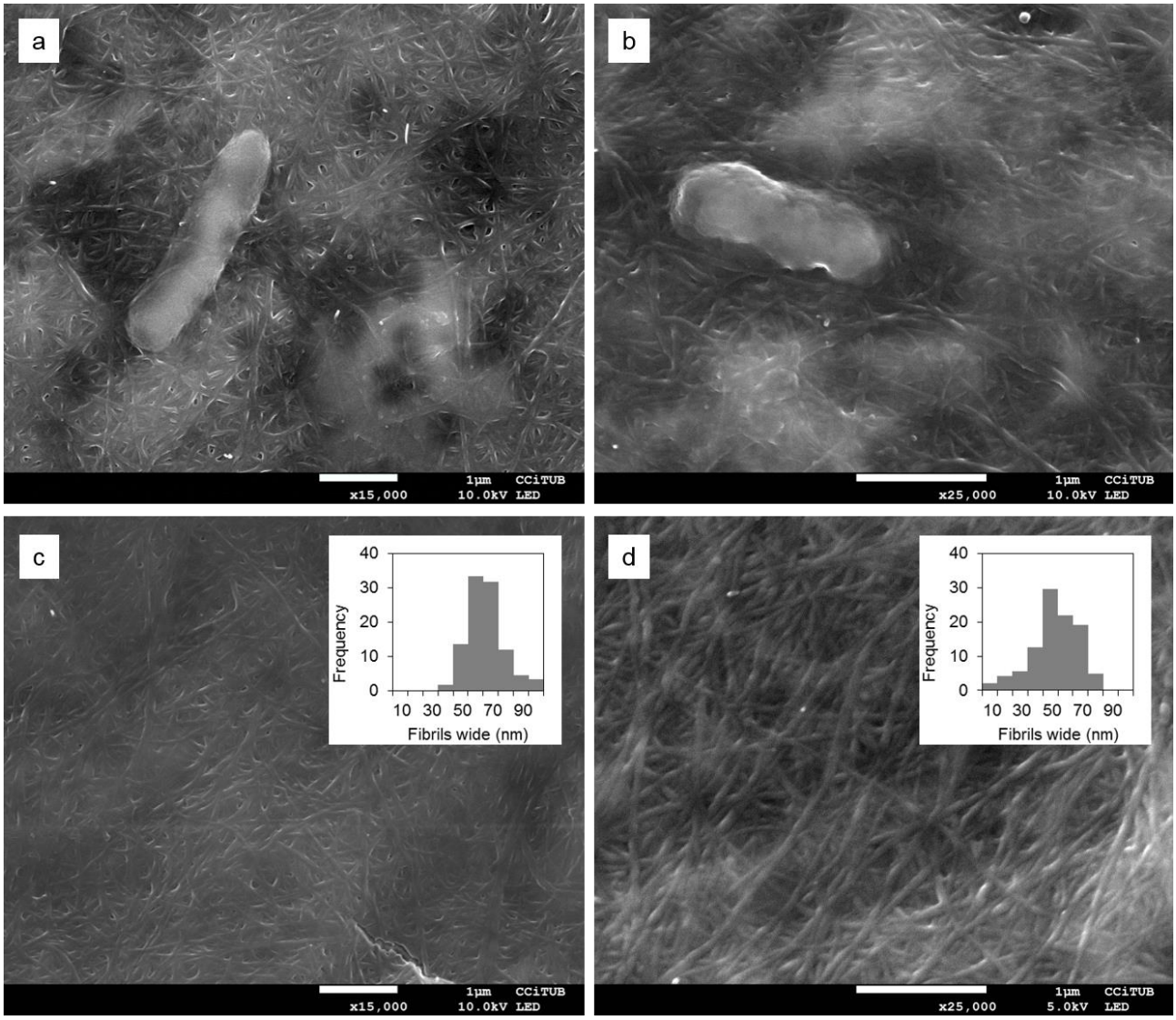

Figure 6. SEM images showing cells of *K. intermedius* JF2 (a) and *K. xylinus* (b), and bacterial cellulose pellicle produced from *K. intermedius* JF2 (c) and *K. xylinus* (d). In the insert of *c* and *d*, diameter size distribution of fibers from *K. intermedius* JF2 and *K. xylinus*, respectively.

FT-IR analysis

Composition and purity of the cellulose produced by *K. intermedius* JF2 was investigated by FT-IR spectroscopy and compared with that of *K. xylinus* reference strain. FT-IR spectra of BC produced by *K. intermedius* JF2 on HS*, K. intermedius* JF2 on HS-glu-man and *K. xylinus* on HS are shown in Figure 7.

The band centred at 899 cm^{-1} characterizing a β-1,4-glycosidic bond, typical of β-linked glucose polymers.[60] The band at 1,050 cm^{-1} could be associated with ether C–O functionalities.[61] The band at 1,100 cm^{-1} is associated with ester C–O bond stretching. The band at 1,160 cm^{-1} is assigned to cellulose C–O–C bridges.[62] The weak band found at 1,330 cm^{-1}, can be ascribed CH2 wagging.[60] The band centered at around 1,420 cm^{-1} could be associated with either CH2 symmetrical bending or surface carboxylate groups. The band at 1,640 cm^{-1} is due to the H–O–H bending vibration of absorbed water molecule.[62] The band at 2,890 cm^{-1} is attributed to C-H stretch for sp^3 carbon (strong). The bands at 3,340 cm^{-1} indicate intermolecular and intramolecular hydrogen bonds. Spectra of cellulose produced by JF2 were very similar to that from *K. xylinus*. Changes were not observed on the absorption spectra of the cellulose produced when JF2 was grown with mannitol. Results suggested that the membranes were free of impurities and that its chemical composition was the same regardless the strain and the culture media assayed. The results of cellulose absorption spectra were very similar to those reported previously.[63,64]

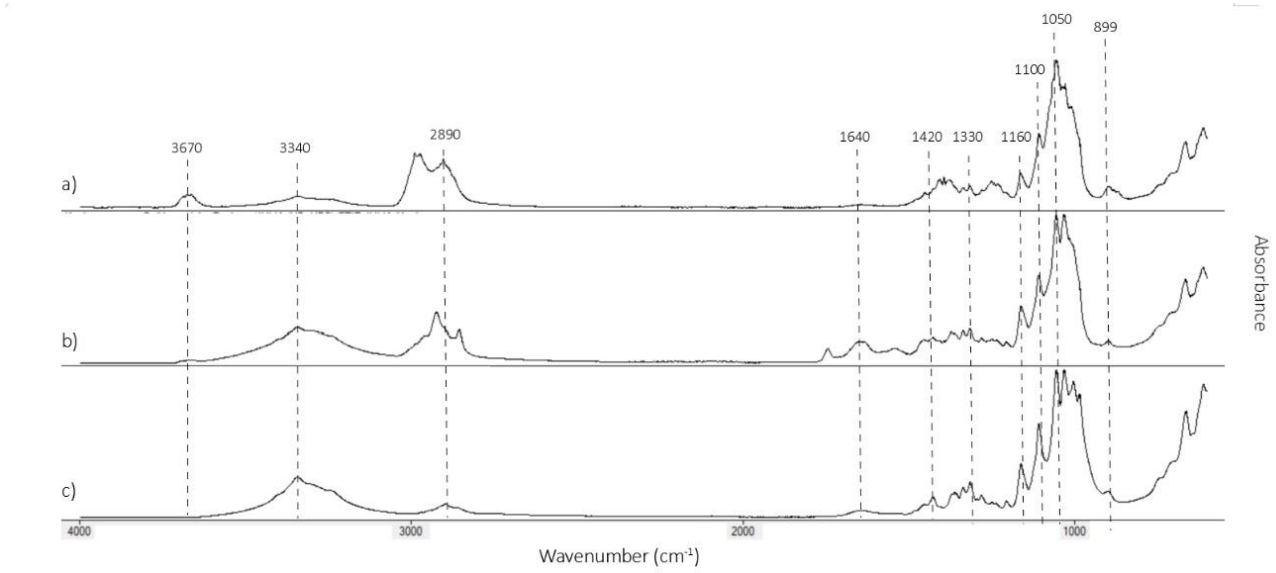

Figure 7. FTIR spectra of BC produced by *K. intermedius* JF2 growing on HS-glu-man (a), *K. intermedius* JF2 growing on HS (b) and *K. xylinus* growing on HS (c).

BC crystallinity

Diffraction patterns of BC obtained from *K. intermedius* JF2 growing on HS and HS-glu-man, and *K. xylinus* grown on HS are shown in Figure 8. The main diffraction angles at 2Θ = 14,59° (1–10), 16,67° (110) and 22,71° (200), correspond to the primary diffraction of the (1-10), (110) and (200) planes of polymorph cellulose I.[65] The diffraction patterns were similar for the three types of samples suggesting that the membranes were composed of cellulose I regardless the bacterial strain and the culture media assayed.

Crystallinity of cellulose can be determined by different methods, and the results are known to be dependent on the method applied. However, results obtained by each particular method are useful for comparing samples identically analyzed.[66] The crystallinity index (CI) of BC pellicles was calculated by Segal method using equation (2). As shown in Table 5, cellulose produced by the new strain *K. intermedius* JF2 presented a higher CI than the reference strain, *K. xylinus*. When the medium was enriched with mannitol, bacterial cellulose from *K. intermedius* JF2 showed higher crystallinity index. It has been described that high CI index confer superior mechanical strength to the fiber of cellulose as well as improved interfacial properties.[67] The higher

CI% of BC pellicles produced by *K. intermedius* JF2 suggests that the process of organization of the glucan chains, upon its synthesis, into cellulose fibrils could be different from that occurring in *K.xylinus*. These results agreed with the difference observed in the diameter of the fibers produced by the two strains.

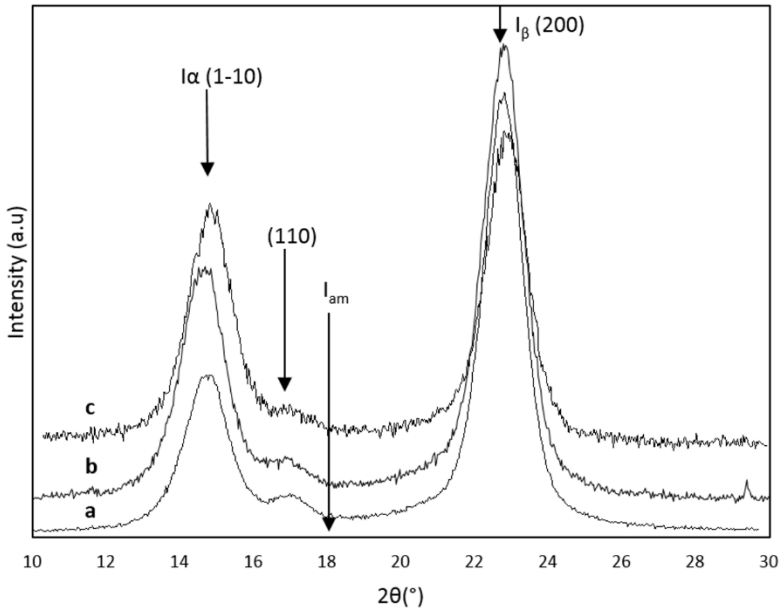

Figure 8. XRD patterns of BC obtained from *K. intermedius* JF2 growing on HS (a), *K. intermedius* JF2 growing on HS-glu-man (b) and *K. xylinus* growing on HS (c).

Table 4. Crystallinity index (CI) of *K. intermedius* JF2 grown on HS, *K. intermedius* JF2 grown on HS-glu-man and *K. xylinus* grown on HS. CI (%) was calculated from empirical equation (1).

***K. intermedius* JF2 (HS-glu-man)**	***K. intermedius* JF2 (HS)**	***K. xylinus***
94.01 ± 0.31	89.90 ± 0.08	84.33 ± 2.4

Water holding capacity (WHC)

WHC of the membranes produce by JF2 and K.xylinus was measured. Dried and weighted BC films were immersed in distilled water for 24 hours, weighted again, and the percentage of water absorbed was calculated. Grown on HS, the WHC of K. intermedius JF2 was 10.1%, lower than that of the reference strain K. xylinus (20.9%). The lowest WHC value was found for membranes produced by K. intermedius JF2 grown on HS-glu-man (7.61%). Interestingly, these results are inversely correlated with those of crystallinity; membranes with higher CI showed less water holding capacity. Huang et al. (2010) found similar results modifying the structure and crystallinity of BC to enhance its ability to rehydrate.

CONCLUSIONS

In this study a novel bacterium capable of producing cellulose was isolated from wine vinegar and identified as a strain of *Komagataeibacter intermedius*, named JF2, based on its physiological characteristics, 16S rDNA sequence and MALDI-TOF mass spectrometry analysis. BC yield production was higher than that of reference strain *K. xylinus*, producing more cellulose in a shorter period of time. JF2 was able to produce cellulose growing with mannitol as the sole carbon source, obtaining better yields than growing with glucose. The produced BC membranes were cellulose I type, free of impurities and with a high index of crystallinity. *Komagataeibacter intermedius* JF2 presents characteristics that make it a suitable candidate as a BC producer at a commercial level for biotechnological applications.

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