Bacterial cellulose produced by a new acid-resistant strain of *Gluconacetobacter* genus

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ABSTRACT

A bacterial strain isolated from the fermentation of Colombian homemade vinegar, *Gluconacetobacter medellensis*, was investigated as a new source of bacterial cellulose (BC). The BC produced from substrate media consisting of various carbon sources at different pH and incubation times was quantified. Hestrin–Schramm (HS) medium modified with glucose led to the highest BC yields followed by sucrose and fructose. Interestingly, the microorganisms are highly tolerant to low pH: an optimum yield of 4.5 g/L was achieved at pH 3.5, which is generally too low for other bacterial species to function. The cellulose microfibrils produced by the new strain were characterized by scanning and transmission electron microscopy, infrared spectroscopy X-ray diffraction and elemental analysis. The morphological, structural and chemical characteristics of the cellulose produced are similar to those expected for BC.

1. Introduction

Cellulose is the most abundant polysaccharide produced in the biosphere. It can be synthesized by plants, animals and microorganisms (Bielecki, Krystynowicz, Turkiewicz, & Kalinowska, 2005). In the latter case, cellulose is mainly produced by Gram-negative bacteria of the *Gluconacetobacter* genus. These bacteria are strictly aerobic and capable of generating cellulose as an extracellular product on static media (at the air-medium interface) at temperatures between 25 and 30 °C and pH from 4 to 7 (Iguchi, Yamanaka, & Budhiono, 2000). Typical carbon sources in the production of bacterial cellulose (BC) include glucose, fructose, sucrose, mannitol, among others. In contrast to the cellulose produced by plants, BC is more crystalline and free from the typical components of the cell wall, namely, lignin, hemicellulose and other biopolymers and extractives. These features make BC an interesting material for nutritional additives, artificial skin, composite reinforcement, electronic paper and for application in other areas where plant cellulose is typically used (Bielecki et al., 2005).

Cellulose-producing species belonging to the *Gluconacetobacter* genus have been reported, where BC synthesis has been strictly linked to the cell metabolism. In fact, the culture conditions have a crucial influence on BC production, in particular regarding factors such as carbon and nitrogen sources, temperature, pH and reactor type (Bielecki et al., 2005; Chawla, Bajaj, Survase, & Singhal, 2009).

In this work, we have investigated the influence of the culture method to produce BC from a new strain, *Gluconacetobacter medellensis* nov., harvested from fermented vinegar from local markets in Medellin, Colombia (Castro et al., 2012). The effect of carbon sources enriched with glucose, sucrose, fructose, mannose, lactose, xylose, glycerol, mannitol, sorbitol and ethanol was investigated as well as incubation pH and time.

BC pellicles obtained from the media that led to the highest yields were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD) and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FT-IR). The characteristics of BC produced and the potential prospects of optimum incubation conditions are discussed in comparison with results from more commonly used bacterial strains.

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2. Materials and methods

2.1. Incubation and BC production

The *G. medellensis* strain from Colombian vinegar culture was purified and classified as a new strain of *Gluconacetobacter* genus (Castro et al., 2012). The strain was cultivated in a standard Hestrin–Schramm medium (HS), *i.e.*, aqueous solution of 2% (w/v) glucose, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.27% (w/v) Na_2HPO_4 and 1.15 g/L citric acid (Hestrin & Schramm, 1954).

The HS medium was modified to investigate the effect of different carbon sources on the production of BC. In the following, such media is referred to as HS-modified. In HS-modified media, glucose was replaced with either of the following sugars: maltose, fructose, cellulose, mannitol, xylose, sucrose or galactose. The carbon source that resulted in the highest BC yield was used to evaluate further the effect of initial pH. This was done by using HS-modified with different concentrations of citric acid buffer. Finally, the optimum incubation time was determined at pH conditions corresponding to the highest bacterial activity.

Experiments were conducted by transferring a colony from HS-agar medium into the respective HS-modified media and static incubation at 28 °C. At the end of incubation, the pellicles were collected, washed with water and treated for 14 h with 5 wt% KOH aqueous solution, followed by rinsing until pH 7 and oven-drying at 70 °C for 48 h between Teflon plates.

2.2. Microfibril morphology

Strips of the BC pellicles were cut, freeze-dried and coated with gold/palladium using an ion sputter coater. The specimens were observed with a Jeol JSM 5910 LV scanning electron microscope operating at 20 kV.

BC microfibrils were homogenized in a Waring blender, diluted in distilled water and sonicated to achieve a good dispersion. Drops of the suspensions were deposited onto glow-discharged, carbon-coated TEM grids and negatively stained with 2 wt% uranyl acetate. The samples were observed with a Philips CM200 microscope operating at 80 kV. The images were recorded on Kodak SO163 films.

2.3. Chemical analyses

Elemental composition (C, H, N, O) of the produced BC was determined by elemental analysis in a CHNS/O Perkin Elmer Serie II 2400. Films of BC microfibrils were dried for 2 h at 100 °C to remove moisture and FT-IR spectra were recorded in ATR mode on a Nicolet 6700 spectrophotometer in the 4000–400 cm^{-1} range, with a resolution of 4 cm^{-1} and after accumulation of 64 scans.

2.4. Structural analysis

Cellulose microfibrils suspensions were centrifuged and the pellets were allowed to dry on a Teflon surface. The resulting films were X-rayed using a Philips PW3830 generator operating at the Ni–filtered CuKα$_1$ radiation wavelength (λ = 0.1542 nm). The films were oriented either perpendicular or parallel to the X-ray beam. Two-dimensional diffraction patterns were recorded on Fujifilm imaging plates and read using a Fujifilm BAS 1800 II bio-imaging analyzer. Diffraction profiles were obtained by integrating the intensity of the 2D diagrams over definite angular sectors.

3. Results and discussion

The cellulose production by *G. medellensis* was investigated by using culture media enriched with different carbon sources. The

Table 1
Amount of bacterial cellulose synthesized by *G. medellensis* in HS-modified media containing various carbon sources.

Carbon source	Dry cellulose production (g/L)
Maltose	0.1
Glucose	3.0
Fructose	0.4
Cellobiose	0.1
Mannitol	0.4
Xylose	0.1
Sucrose	2.0
Galactose	0.1

mass of BC produced per liter of medium was determined after 8-day incubations in static culture at 28 °C (Table 1). The BC yield from glucose-rich sources was the highest (3 g/L), whereas the sucrose-rich medium yielded 2 g/L BC and fructose- and mannitol-rich media both produced 0.4 g/L BC. Maltose, cellobiose, xylose and galactose appeared to be less suitable as carbon sources for BC production by this new strain.

Together with other carbohydrates, glucose has often been used as a common carbon source for cellulose production by *Gluconacetobacter* strains (Castro et al., 2012). However, D-glucose, unlike other sugars, acts not only as an energy source but also as an ideal precursor for the assembly of the highly structured cellulose polymer. Its consumption during bacterial incubation is nearly total and in quantitative agreement with the amount of BC produced (Klemm, Shumann, Udhardt, & Marsch, 2001; Masaoka, Ohe, & Sakota, 1993).

It has been reported that an optimum pH for BC production depended on the bacterium strain, but has usually been ascribed to be within a neutral to slightly acidic pH range (Bielecki et al., 2005). A reasonable question is therefore if similar conditions favor BC production in the presented system. As such, the HS medium modified with glucose as a source of carbon was used to evaluate the effect of pH on BC yield. Fig. 1 shows the amount of BC produced as a function of pH. After a 8-day incubation, the BC production was confirmed in all media tested. However, in contrast to previous findings for bacteria of the same genus, *G. medellensis* strain reached a maximum production in acidic medium (4.5 g/L at pH 3.5). Interestingly, this pH level is the one measured when BC production from other species is exhausted, as the process becomes less efficient or BC production is stopped (Gromet-Elhanan & Hestrin, 1963; Klemm et al., 2001).

The glucose in the culture medium is expected to be converted into (keto) gluconic acids during BC production, thus lowering the pH and limiting cell viability (Masaoka et al., 1993; Vandamme, De Baets, Vanbaelen, Joris, & De Wulf, 1998). Several attempts have been made to harvest or engineer strains that are resilient to low pH media during BC biosynthesis (De Wulf, Joris, & Vandamme, 1996; Park, Song, & Kim, 1999). In addition, some co-adjuvants such as acetates, citrates and succinates have been applied in the culture media to maximize the production of cellulose at low pH. These chemicals are believed to act by a mechanism in which they are oxidized and thus reduce the consumption of sugars in oxidative reactions (Dudman, 1959; Gromet-Elhanan & Hestrin, 1963). Similarly, the citric acid buffer used in the HS medium is expected to oxidize and aid the glucose substrate for BC synthesis. Therefore, for typical bacterial strains, maintaining the culture pH within the optimum range by addition of co-adjuvants and citric acid buffer is critical to ensure high BC yields. Remarkably, the new strain investigated here yields a relatively large amount of BC even in pH 3 media, indicating its tolerance to extremely acidic environments while optimally producing cellulose. Such situation is highly desirable in industrially relevant fermentation processes for BC production with the added advantage that it limits microbial contamination
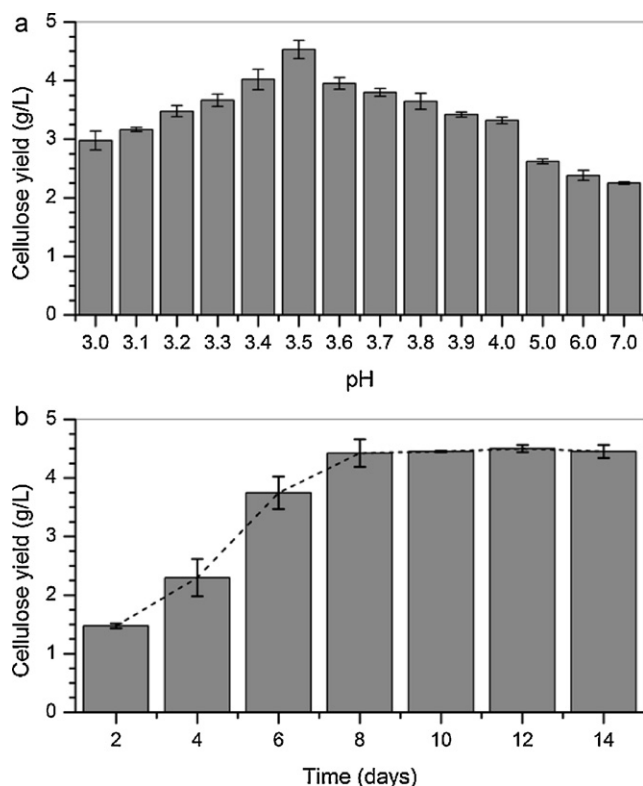
Fig. 1. BC yield measured as a function of pH (a) and time (b) for *G. medellensis* incubated in HS-modified medium with glucose as carbon source. In (b), the culture media were acidified at pH 3.5 and the temperature was 28 °C.


Fig. 2. (a) SEM image of a BC pellicle produced by *G. medellensis* in HS-medium acidified to pH 3.5 and incubated for 8 days. (b) Close-up on a synthesizing bacterium attached to cellulose ribbons. (c) TEM micrograph of negatively stained BC ribbons.

since most microorganisms are not viable in such low pH media (Toda, Asakura, Fukaya, Entani, & Kawamura, 1997).

The influence of the incubation time in BC production was investigated in incubations with initial pH of 3.5 (Fig. 1b). Typically, an easily visible pellicle was formed on top of the media after incubation for 2 days. The amount of cellulose produced increased until 8 days. A plateau in production was observed after this time. It is expected that during the initial stage, the bacterial population increased at the expense of the consumption of oxygen dissolved in the medium. Therefore, the bacteria located close to the surface of the fluid medium began to produce cellulose in the form of layers. In addition, younger cells were produced, thereby allowing cellulose production to continue in the uppermost layer of the film, at the pellicle/air interface (Borzani & Souza, 1995; Masaoka et al., 1993). In summary, the largest BC yield by *G. medellensis* took place in HS-modified medium with glucose as carbon source, acidified to pH 3.5 and incubated for 8 days. As the production of cellulose by this bacterium did not affect the final pH, after 8 days, the cellulose could be aseptically taken out and the medium could be re-used for additional incubation cycles. Thus, a sustainable production of BC in integrated processes whereby the resource consumption is minimized can be ensured with this new bacterial strain.

The surface pellicles formed by *G. medellensis* in HS-modified medium were observed by SEM (Fig. 2a). Bacillary-shaped cells are observed entrapped in the network, and a fibril structure is observed directly arising from the cell surface (Fig. 2b). The microfibrils in the network are uniformly and randomly oriented, probably because the organism doubles the microfibril-synthesizing sites prior to division, and both parent and daughter sites are active just before division, synthetizing a microfibrilar ribbon of constant dimensions.

It has been reported that the size of this ultrastructure (in the nanometer scale) is a critical factor that determines the unique
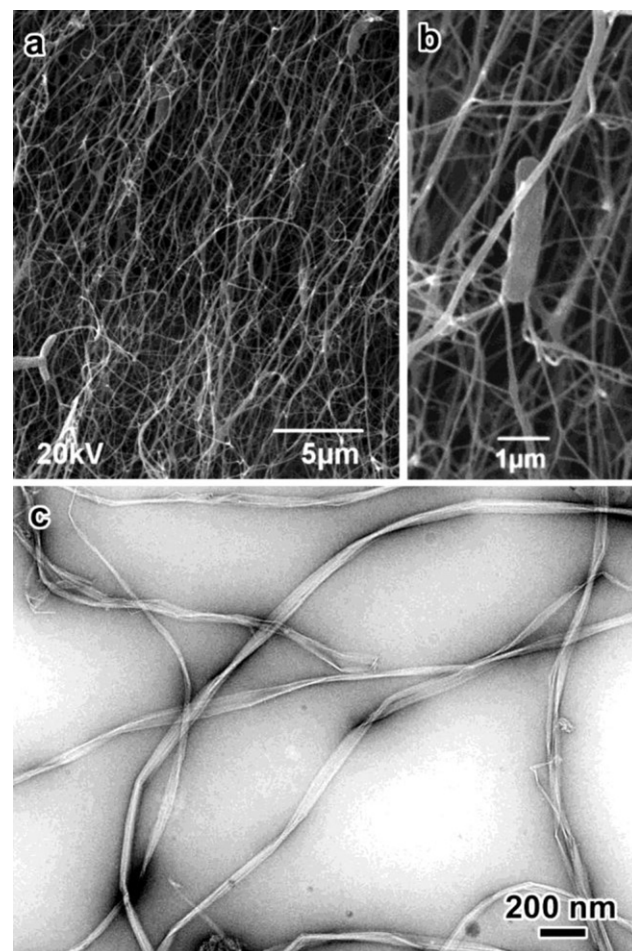
properties of reticulated bacterial cellulose (Brown, Willison, & Richardson, 1976). The synthesized microfibrils were thus observed by TEM (Fig. 2c). The microfibrils are clearly ribbon-shaped with a width ranging between 40 and 70 nm. Such observations agree with the typical morphology reported for BC (Yamanaka, Ishihara, & Sugiyama, 2000).

Elemental analysis was carried out as well after purification. Carbon and hydrogen contents of 44.2 ± 1.6 and $6.3 \pm 0.25\%$, respectively, were determined. This elemental composition is in agreement with that of pure cellulose and with compositions reported by other investigators (Klemm et al., 2001; Yoon, Jin, Kook, & Pyun, 2006). Some nitrogen was detected in the cellulose simple ($0.39 \pm 0.04\%$) and ascribed to amino acids from the residual bacteria after purification.

The FT-IR spectrum from *G. medellensis* BC as well as the reference spectrum for bacteria are shown in Fig. 3. Spectrum 3a shows the typical bands reported for BC (Shirk & Greathouse, 1952). However, the peak at 1735 cm^{-1} assigned to C=O group in proteins and lipids and the peak at 1538 cm^{-1}, which corresponds to protein amide II absorption appear in the spectrum of BC after KOH treatment. These bands are also found in the spectrum of the bacterium (spectrum 3b), alongside a broad band from 3700 to 3000 cm^{-1} corresponding to O—H of hydroxyl groups and N—H of amine groups, as well as bands from 2959 to 2850 cm^{-1} from fatty acids, at 1695 cm^{-1} resulting from amine I and from 1460 to 1000 cm^{-1} corresponding to amine III and phosphates. The latter band may be present in the spectrum of bacterial cellulose but
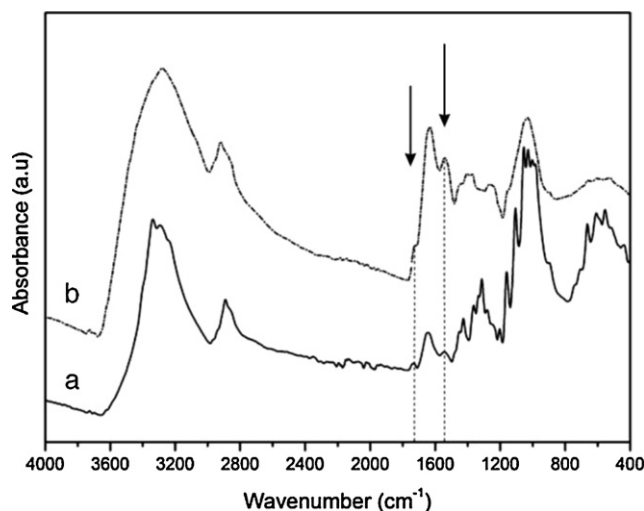
Fig. 3. ATR-FT-IR spectra of a film of cellulose produced by *G. medellensis* (a) and of the total cellular material (b). Bands arising from residual cellular material after cellulose purification are indicated by arrows. Band assignment is detailed in the text.

Fig. 4. X-ray diffraction diagrams recorded from *G. medellensis* BC films oriented perpendicular (a) and parallel (b) to the X-ray beam; (c) corresponding equatorial profiles. The indexing is that of cellulose Iα (Sugiyama et al., 1991).

is superimposed with the characteristic bands of cellulose (Gea et al., 2011; Schmitt & Flemming, 1998). Other bands at 1375 cm^{-1} (C—H bending), 1335 cm^{-1} (O—H in-plane bending), 1315 cm^{-1} (CH2 wagging), 1277 cm^{-1} (C—H bending) and 1225 cm^{-1} (O—H in-plane) indicate the presence of crystalline regions within the structure (Nelson & O'Connor, 1964).
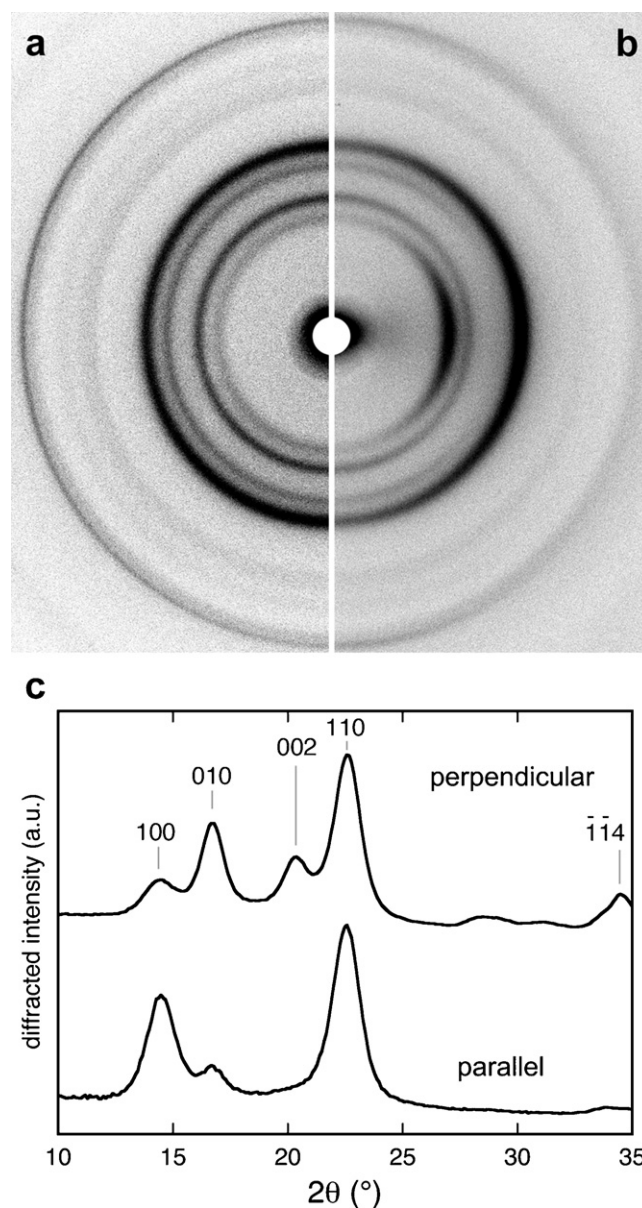
FT-IR spectroscopy was also used to determine the ratio of allomorphs Iα and Iβ (Imai & Sugiyama, 1998) and to calculate the infrared crystallinity index of Nelson and O'Connor (1964). The Iα/Iβ ratio was *ca.* 0.74 indicating that *G. medellensis* BC is richer in Iα polymorph. The infrared crystallinity index was around 0.65, similar to that reported for BC from other sources (Imai & Sugiyama, 1998; Pecoro, Manzani, Messaddeq, & Ribeiro, 2008, chap. 17).

The powder and fiber XRD diagrams recorded with the film oriented perpendicular and parallel to the X-ray beam, respectively, are shown in Fig. 4a and b. The rings and arcs characteristic of cellulose I are observed in both diagrams. The 5 main peaks were assigned to the (1 0 0), (0 1 0), (0 0 2), (1 1 0) and ($\bar{1}$ $\bar{1}$ 4) crystallographic planes of the triclinic Iα allomorph (Sugiyama, Persson, & Chanzy, 1991), corresponding to diffraction angles of 14.4, 16.7, 20.3, 22.5 and 34.4°, respectively. The intensity of the 1 0 0 reflection is larger than that of the 0 1 0 one when the film is parallel to the X-ray beam and the effect is reversed in the perpendicular orientation. This reveals a strong uniplanarity that is due to the fact that the cellulose ribbons are preferentially oriented parallel to the film surface during drying.

Overall, the new acid-resistant bacterial strain is capable of producing cellulose at low pH, at high yields and under conditions favorable for recycling of the medium yielding BC with characteristics similar to those reported for BC from other species of the genus *Gluconacetobacter*.

4. Conclusions

The production on bacterial cellulose by a new bacterial strain was evaluated. HS-modified media were used to evaluate BC production with different carbon sources, pH and incubation time. Glucose as main carbon source was found to produce the highest BC yields, and optimum pH of 3.5 was determined after 8 days of incubation. The initial pH for maximum cellulose production (yield of 4.5 g/L) was distinctively low compared to the pH typically used in incubation with bacteria of this genus. Overall, the new

G. medellensis strain is acid-resistant and can be used sustainable in multiple incubation cycles.

References

Bielecki, S., Krystynowicz, A., Turkiewicz, M., & Kalinowska, H. (2005). Bacterial cellulose. In A. Steinbuchel (Ed.), *Biotechnology of polymer: From synthesis to patents* (pp. 381–434). Munster, Germany: Wiley-VCH, Verlag GmbH.
Borzani, W., & Souza, S. J. (1995). Mechanism of the film thickness increasing during the bacterial production of cellulose on non-agitated liquid media. *Biotechnology Letters*, *17*, 1271–1272.

Brown, R. M., Willison, J. H. M., & Richardson, C. L. (1976). Cellulose biosynthesis in *Acetobacter xylinum*: Visualization of the site of synthesis and direct measurement of the *in vivo* process. *Proceedings of the National Academy of Sciences of the United States of America, 73*, 4565–4569.

Castro, C., Zuluaga, R., Cleenwerck, I., Trcek, J., De Vos, P., Putaux, J.-L., et al. (2012). *Gluconacetobacter medellensis* sp. nov., a novel cellulose-producing bacterium isolated from homemade fruit vinegar and reclassified specie of *Gluconacetobacter xylinum*. *International Journal of Systematic and Evolutionary Microbiology*, under review.

Chawla, P. R., Bajaj, I. B., Survase, S. A., & Singhal, R. S. (2009). Microbial cellulose: Fermentative production and applications. *Food Technology and Biotechnology, 47*, 107–124.

De Wulf, P., Joris, K., & Vandamme, E. J. (1996). Improved cellulose formation by an *Acetobacter xylinum* mutant limited in (keto)gluconate synthesis. *Journal of Chemical Technology and Biotechnology, 67*, 376–380.

Dudman, W. F. (1959). Cellulose production by *Acetobacter acetigenum* in defined medium. *Microbiology, 21*, 327–337.

Gea, S., Reynolds, C. T., Roohpour, N., Wirjosentono, B., Soykeabkaew, N., Bilotti, E., et al. (2011). Investigation into the structural, morphological, mechanical and thermal behaviour of bacterial cellulose after a two-step purification process. *Bioresource Technology, 75*, 18–22.

Gromet-Elhanan, Z., & Hestrin, S. (1963). Synthesis of cellulose by *Acetobacter xylinum* VI. Growth on citric acid-cycle Intermediates. *Journal of Bacteriology, 85*, 284–292.

Hestrin, S., & Schramm, M. (1954). Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. *Biochemical Journal, 58*(2), 345–352.

Iguchi, M., Yamanaka, S., & Budhiono, A. (2000). Review: Bacterial cellulose – A masterpiece of nature's arts. *Journal of Materials Science, 35*, 261–270.

Imai, T., & Sugiyama, J. (1998). Nanodomains of Iα and Iβ cellulose in algal microfibrils. *Macromolecules, 31*, 6275–6279.

Klemm, D., Shumann, D., Udhardt, U., & Marsch, S. (2001). Bacterial synthesized cellulose – Artificial blood vessels for microsurgery. *Progress in Polymer Science, 26*, 1561–1603.

Masaoka, S., Ohe, T., & Sakota, N. (1993). Production of cellulose from glucose by *Acetobacter xylinum*. *Journal of Fermentation and Bioengineering, 75*, 18–22.

Nelson, M. L., & O'Connor, R. T. (1964). Relation of certain infrared bands to cellulose crystallinity and crystal lattice type. Part II. A new infrared ratio for estimation of crystallinity in cellulose I and II. *Journal of Applied Polymer Science, 8*, 1325–1341.

Park, S. T., Song, T. S., & Kim, Y. M. (1999). Effect of gluconic acid on the production of cellulose in *Acetobacter xylinum* BRC5. *Journal of Microbiology and Biotechnology, 9*, 683–686.

Pecoro, E., Manzani, D., Messaddeq, Y., & Ribeiro, S. (2008). Bacterial cellulose from *Glucanacetobacter xylinus*: Preparation, properties and applications. In M. N. Belgacem, & A. Gandini (Eds.), *Monomers, polymers and composites from renewable resources* (pp. 369–383). Amsterdam, Netherlands: Elsevier.

Schmitt, J., & Flemming, H. (1998). FTIR-spectroscopy in microbial and material analysis. *International Biodeterioration and Biodegradation, 41*, 1–11.

Shirk, H., & Greathouse, G. (1952). Infrared spectra of bacterial cellulose. *Analytical Chemistry, 24*, 1774–1775.

Sugiyama, J., Persson, J., & Chanzy, H. (1991). Combined infrared and electron diffraction study of the polymorphism of native cellulose. *Macromolecules, 24*, 2461–2466.

Toda, K., Asakura, T., Fukaya, M., Entani, E., & Kawamura, Y. (1997). Cellulose production by acetic acid-resistant *Acetobacter xylinum*. *Journal of Fermentation and Bioengineering, 84*, 228–231.

Vandamme, E., De Baets, S., Vanbaelen, A., Joris, K., & De Wulf, P. (1998). Improved production of bacterial cellulose and its application potential. *Polymer Degradation and Stability, 59*, 93–99.

Yamanaka, S., Ishihara, M., & Sugiyama, J. (2000). Structural modification of bacterial cellulose. *Cellulose, 7*, 213–225.

Yoon, S. H., Jin, H.-J., Kook, M.-C., & Pyun, Y. R. (2006). Electrically conductive bacterial cellulose by incorporation of carbon nanotubes. *Biomacromolecules, 7*, 1280–1284.