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Short communication

Vitamin C enhances bacterial cellulose production in *Gluconacetobacter xylinus*

Sherif M.A.S. Keshk[a,b,*]

[a] King Khalid University, Faculty of Science, Chemistry Department, P.O. Box 9004, Abha 61413, Saudi Arabia
[b] Ain-Shams University, Institute of Environmental Studies and Research, Basic Science Department, Abbassia, Cairo 11566, Egypt

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ABSTRACT

Influence of vitamin C (ascorbic acid) on bacterial cellulose (BC) production and crystal structure was studied using four strains of *Gluconacetobacter xylinus* (ATCC 10245, IFO 13693, 13772 and 13773). BC productivity of all strains was increased in presence of vitamin C (0.5% w/w), the average BC production reached 0.47 g/30 ml compared with 0.25 g/30 ml without vitamin C. Enhanced productivity is associated with a decrease in gluconic acid concentration that is produced from *Gluconacetobacter xylinus* during BC production. X-ray results showed that the crystallinity index of BC produced in presence of ascorbic acid was the lowest with remarkable change in d-spacing. These results were confirmed by using solid state ^{13}CNMR. The increase in BC yield in presence of vitamin C is due to its antioxidant behavior and confirms our past work on lignosulfonate influence on BC.

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1. Introduction

Bacterial cellulose is produced extracellularly by bacteria of the genus *Glucanobacter* cultivated in a culture medium containing carbon sources such as sugar and nitrogen sources. Bacterial cellulose is in ribbon form composed of microfibrils, they are formed on the surface of a bacterium as a linearly ordered array of terminal complexes (Brown, Willison, & Richardson, 1976; Zaar, 1977). Each terminal complex produces nanometer-sized subfibrils that are crystallized into a microfibril. Several microfibrils are aggregated into a single ribbon. The size of the ribbons has been reported to be in the range of 3–4 nm to 70–80 nm (Zaar, 1977; Brown et al., 1976) and seems to be constant among various strains of *Glucanobacter* and unchanging under culture conditions. Bacterial cellulose production has been reported to be enhanced by addition of a small amount of cellulase complex (cellulase complex having endo-glucanase, exo-cello-bio-glucosidase and β-glucosidase activities and cellulose binding) to the culture broth (Delmer & Yehudit, 1980). Tonouchi, Tahara, Tsuchida, Yoshinaga, & Beppu (1995) examined cellulose production by *Gluconacetobacter xylinus* using a single species of β-1,4-endoglucanase and its active site mutant. This still had cellulose-binding ability, but was devoid of the enzyme activity, to identify the effect of the cellulose-binding ability on cellulose production. They found that the endoglucanase activity itself, not the cellulose-binding ability, enhanced bacterial cellulose production. The addition of small amount cellulase does not affect the degree of polymerization of BC but enhances the production rate (Tonouchi et al., 1995). It has been found that the addition of vitamin C into culture medium remarkably enhanced the efficiency for cellulose production of *Gluconacetobacter xylinus* (Premjet, Ohtani, & Sameshima, 1994; Keshk & Sameshima, 2006; Keshk, 2011). The improvement of BC yield in presence of lignosulfonate brought about the decrease of gluconic acid concentration due to antioxidant nature of lignosulfonate (Keshk & Sameshima 2006; Keshk, 2011). Lignosulfonate not only increases the yield of BC but also increases the crystallinity index. However, lignosulfonate does not interfere with the planes of the cellulose and its steric hindrance (Keshk & Sameshima, 2006; Keshk, 2006). Improving the production and the physical properties of BC is important. Physical properties of BC are influenced by the ultra-structure of cellulose, especially by the size of cellulosic ribbons. It was, therefore, of interest to attempt to change the size of the ribbons. We investigated the possibility of changing the ribbon structure by adding chemical reagents (direct dyes or lignosulfonate to the culture broth of *Glucanobacter* (Keshk, 2006, 2011). The strategy was to alter the morphology of the bacteria that produce cellulose. The shape of the cells affects ribbon formation because the biosynthesis of the ribbons occurs at loci on the cell surface. Ascorbic acid is a naturally occurring organic compound with antioxidant properties. Ascorbic acid, also known as vitamin C, is a water-soluble vitamin

* Correspondence at: Ain-Shams University, Institute of Environmental Studies and Research, Basic Science Department, Abbassia, Cairo 11566, Egypt.
Tel.: +966172266786/966564433700; fax: +20 96672266768.
E-mail addresses: keshksherif@gmail.com, sherifkeshk@yahoo.com

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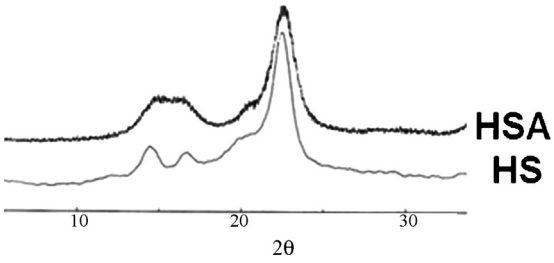

Fig. 1. X-ray pattern of bacterial cellulose in presence of vitamin C (HSA) and without it (HS).

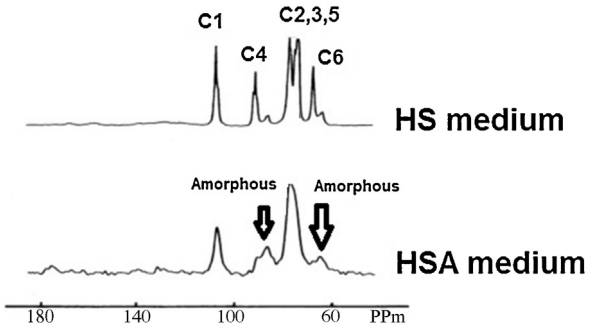

Fig. 2. Cp/MAS of bacterial cellulose in presence of vitamin C (HSA) and without it (HS).

necessary for normal growth, development and repair of damaged tissues in the body. It is essentially an antioxidant which is needed for the prevention of some of the damage done when one is exposed to cigarette smoke, radiation or when the body breaks down food. In present work, I am going to confirm the antioxidant behavior of lignosulfonate using ascorbic acid. Moreover, the crystal structure of BC produced in presence of ascorbic acid will be studied using both X-ray diffractometer and solid state ^{13}C-NMR. Figs. 1 and 2.

2. Materials and methods

American Type Culture Collection (ATCC) is the supplier of the *Gluconacetobacter xylinus* ATCC 10245, whereas Institute of Fermentation in Osaka (IFO) is the supplier of *Gluconacetobacter xylinus* IFO 13693, 13772, and 13773.

2.1. Culture media and conditions

Two types of culture media were used, namely the Hestrin–Schramm culture medium (Hestrin & Schramm, 1954), in presence of glucose (HS) and in presence of 0.5% ascorbic acid (HSA). All cultures were incubated statically at 28 °C in the liquid medium at pH 6 for 1 week.

2.2. Pellicles production and purification

BC membrane was produced by different *Gluconacetobacter xylinus* strains in 30 ml of HS and HSA media at 28 °C for 7 days using 90-mm (i.d.) petridishes. After incubation, the pellicles produced on the surface of each medium were harvested and washed with water, hot 2% sodium dodecyl sulfate (SDS) and water successively, then dried on a Teflon plate.

2.3. Measurement of gluconic acid concentration by BTB method

Both culture media of HS and HSA were centrifuged at 4500 rpm for 30 min and the supernatants were filtered through a 0.22 μm filter (MILLEX-GS, Carrigtwohill, Co. Cork, Ireland). The gluconic acid concentration of the filtrate was measured by a high performance liquid chromatography (HPLC) (column: GL-C610 Hitachi Co., Ltd., Japan), eluted with 3 mM perchloric acid at flow rate of 1.0 ml/min at a column temperature of 63 °C and detected using spectrophotometer (VIS 440 nm) by bromo-thymol blue (BTB) method.

2.4. Wide angle X-ray diffractometry

The thick samples of BC were prepared according to Kai and Keshk (1999). The reflectogram of the samples was recorded at room temperature with Rigaku Print 2200 V series using Ni-filtered Cu Kα radiation (λ = 1.54 Å). The operating voltage and current were 40 Kv and 30 mA, respectively. Crystallinity was calculated from the reflected intensity data using Segal, Creely, Martin, & Conrad, 1959 method according to Eq. (1).

$$(\text{C.I.}) = (I_{020} - I_{\text{am}})/I_{020} \quad (1)$$

I_{020} is the maximum intensity of the lattice diffraction and I_{am} was the intensity at $2\theta = 18^{\circ}$.

2.5. Solid state ^{13}C NMR spectroscopy

The CP/MAS ^{13}C NMR spectra were recorded (at 292 ± 1 K) on the Joel CMX-300 instrument operating at 7.0 T. A double air-bearing probe and a zirconium oxide rotor were used. The MAS rate was in the 4–5 kHz range. Acquisition was performed with a standard CP pulse sequence using a 5 s proton 90 pulse, a 1200 s contact pulse and 3 s delays between repetitions. Adamantine was used as an external standard for the chemical shift scale relative to tetra methyl silane. The crystallinity of BC was calculated from the C_4 crystalline part in the resolution enhanced spectra after deconvolution

3. Results and discussion

BC production using vitamin C in HS medium was evaluated in this experiment. We used six strains of *Gluconacetobacter xylinus*, ATCC 10245 and IFO 13693, 13772 and 13773. The presence of vitamin C in HS medium increased the yield of BC in all strains Table 1. *Gluconacetobacter xylinus* IFO 13693 and 13773 were the best producers (0.53 g/30 ml), whereas the strain ATCC 10245 showed the best yield increase ratio (1.52), Table 1. The deficiency of BC production in case of HS medium has been explained by the formation of the D-gluconic and D-2-keto-gluconic acid and subsequently the pH of the medium drops to the sub optimal levels of cell viability (Keshk & Sameshima, 2006). As shown in Table 1, the average pH is 3.34 in case of HS medium but in HSA medium is 4.62 (Table 1). These results matched with our previous results (Toda, Asakura, Fukaya, Entani, & Kawamura, 1997; Keshk & Sameshima, 2006), in which we studied the effect of the medium pH on the cellulose yield and the maximum cellulose yield recorded at pH = 4.48 after incubation. HPLC results showed that the concentration of gluconic acid in HSA is lower than that in HS media out of all strains as shown in Table 1, the enhancement of BC yield in presence of vitamin C brought about the decrease of gluconic acid concentration (from 6.35 to 2.32). To check the reason of discernment gluconic acid concentrations, we put 0.01 mmol of vitamin C with 0.01 mmol of gluconic acid in presence of 1 ml acetic acid for 3 days and 7 days at 28 °C, and found that no change in gluconic acid concentration was detected by HPLC. This indicates that the ester formation between vitamin C and gluconic acid is not the case. Vitamin C could be served as an antioxidant and the gluconic acid formation can be reduced. The average crystallinity index calculated from X-ray diffractometer of BC produced in HSA from all strains is lower

Table 1
Bacterial cellulose production of four *Gluconacetobacter xylinus* strains in the medium of Hestrin–Schramm (HS) and in presence of 0.5% vitamin C (HSA).

Strain/parameter	HS medium			HSA medium			Yield ratio
	Yield (g/30 ml)	Gluconic acid (μg/20 μl	Final pH	Yield (g/30 ml)	Gluconic acid (μg/20 μl)	Final pH	HSA/HS
10245	0.23	5.43	2.98	0.35	2.50	3.89	1.52
13693	0.27	7.41	3.40	0.48	2.43	4.82	1.78
13772	0.25	5.73	3.50	0.45	2.02	4.85	1.80
13773	0.30	6.84	3.50	0.58	2.32	4.90	1.93

The average yield of three replicate cultures after cultivation for 7 days is expressed as the weight of BC (mg) per 30 ml of culture medium.

Table 2
Reflective angle, d-spacing and crystallinity index (C.I.) of BC productivity from HS and HSA media with different strains.

Strains	C.I.		$2\theta^{\circ}$/d (1–10)		$2\theta^{\circ}$/d (110)		$2\theta^{\circ}$/d (020)	
	HS	HSA	HS	HSA	HS	HSA	HS	HSA
10245	82.0	65.0	14.56 (6.07)	14.44 (6.08)	16.88 (5.26)	16.74 (5.29)	22.78 (4.90)	22.64 (4.32)
13693	85.0	68.0	14.42 (6.13)	14.46 (6.12)	16.70 (5.30)	16.78 (5.28)	22.62 (4.92)	22.66 (4.40)
13772	86.0	62.0	14.66 (6.03)	14.52 (6.09)	16.96 (5.22)	16.80 (5.27)	22.92 (4.88)	22.70 (3.91)
13773	86.7	64.3	14.46 (6.12)	14.48 (6.11)	16.78 (5.27)	16.76 (5.28)	22.68 (4.91)	22.70 (3.96)

than those from HS media (Tables 1 and 2). Keshk and Sameshima (2006) found that, when lignosulfonate is added to BC it enhances the crystallinity index, and it was explained by the physical cross linkage due to the addition of lignosulfonate which favored the ordered structure of the molecules (Keshk & Sameshima, 2006). Iwata et al. (1998) studied the affinity of lignin-carbohydrate complexes for BC and found that lignin moiety did not directly take part in the formation of cellulose composite. Moreover, our pervious results showed that the presence of direct dyes as fluorescent brighteners or direct blue and lignosulfonate increase the solubility, the steric effect and decrease the affinity toward the cellulose chain (Kai & Keshk, 1998, 1999; Keshk & Sameshima, 2006; Keshk, 2006, 2011). In present study, vitamin C has much lower molecular weight ($C_6H_8O_6$, 176.12) and less steric hindrance than those of direct dyes and lignosulfonate. Moreover, the d-spacing of BC planes from both media (HS and HAS) has remarkable differences (Table 2). So, the discernment in crystallinity index due to addition of vitamin C can be explained by the discernment of hydrogen bonding between the cellulose chains. vitamin C affinity toward nascent structure of BC is the highest among both direct dyes and lignosulfonate owing to the lowest crystallinity index and difference on d-spacing from X-ray measurements.

4. Conclusions

The presence of vitamin C in HS medium enhances BC production by reducing the production of gluconic acid. The discernment of crystallinity index of BC in presence of vitamin C might be caused by the discernment of inter-hydrogen bonding between the cellulose sheets due to presence of vitamin C between cellulose planes. Vitamin C, however, interferes the planes of the cellulose possibly due to its high water solubility, low molecular weight and less steric hindrance.

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