Characteristic variations of bacterial nanocellulose incorporated with different cellulosic additives

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Research Article

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Abstract

Bacterial cellulose (BNC) is a promising biocompatible material. Improvements of the BNC production and its surface characteristics should be attained to make it commercially viable. In this study, in-situ fermentation and production of BNC by addition of different cellulosic substrates such as Avicel and carboxymethylcellulose (CMC) were performed using *Komagataeibacter* sp. SFCB22-18. The addition of cellulosic substrates improved BNC production by a maximum of about 5 times and slightly modified its structural properties. The changes in morphological and structural changes of BNC were investigated by using Fourier transform-infrared spectroscopy (FT-IR) spectroscopy, X-ray diffraction (XRD), and scanning electron microscopy (SEM). Furthermore, a unique biological analysis approach for quantifying the surface crystallinity of the BNC surface was applied using a type-A cellulose-binding protein, *Ct*CBD3. This result shows that since Avicel and CMC may attach on the microfibrils during the production or crystallization of BNC, cellulose-binding protein could be a feasible option for identifying the crystalline properties of BNC with high sensitivity.

Introduction

Cellulose, which is a linear chain of glucose molecules connected by β-1,4-glycosidic linkages, is highly representative of renewable polymers in nature [1]. Cellulose is a biocompatible, biodegradable, and renewable resource to which much attention has been paid, not only as a biopolymer capable of serving as an alternative to plastics in the chemical industry, but also as a medical material for tissue engineering and carrying drugs in the medical industry [2–6]. Furthermore, the derivatization of the cellulose surface can considerably improve its functionalities, broadening the applications of cellulose in numerous industries [7–10].

Lignocellulosic biomass are the most representative source of cellulose; however, since hemicellulose and lignin are incorporated, severe and complex pretreatment processes with bleaching are necessary to obtain pure cellulose, incurring a high operation cost. Therefore, microbial production of pure cellulose (i.e., bacterial cellulose; BNC) has been widely studied as an alternative [2, 3, 11–13]. The approach to producing cellulose using bacteria has shown various advantages, such as a high purity and a high specific surface area.

Many researchers have tried to produce BNC by incorporating different additives (Supplementary Table 1). During BNC synthesis, the additives may improve BNC production or its functional properties [2, 7–9, 14]. These alterations can enable BNC to be used in a variety of industries. Meanwhile, the crystalline structure of cellulose is highly related to the final material's strength [15]; thus, it is important to elucidate changes in this crystallinity. Various approaches to measuring the crystallinity of cellulose have been adopted thus far, and recent studies show that cellulose-binding protein (CBD) may become a more sensitive means of measuring crystallinity changes in cellulose [16, 17]. Since BNC is known to be one of the most crystalline polymers, measuring changes in the crystallinity of modified BNC could become a

vital clue in proposing applications. To date, no studies have been investigating the crystallinity of BNC using CBD.

In this study, we investigated the effects of the addition of cellulosic substrates upon the production and properties (i.e., especially the crystalline properties) of BNC using *Komagataeibacter* sp. SFCB22-18 isolated from ripened persimmons [18]. We also investigated the changes in various structural properties via scanning electron microscopy (SEM), Fourier-transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), and the use of a type-A cellulose-binding protein, *Ct*CBD3. This study provides fundamental information on the effects of the addition of different types of cellulosic substrates during fermentation upon the production and structural modification of BNC.

Materials And Methods

Strain and chemicals

Komagataeibacter sp. SFCB22-18, as obtained from ripened persimmons, was used in this study [18]. The strain was stored at -80°C in a 50% (v/v) glycerol solution. CMC and Avicel were purchased from Sigma-Aldrich (St. Louis, Mo, USA). The strain was routinely cultivated in Hestrin–Schramm modified (HSM) medium containing 30 g glucose, 25 g yeast extract, 2.7 g Na_2HPO_4, 2.4 g acetic acid, and 5 g ethanol per L.

Production and purification of BNC

BNC pellicles were produced and purified by following previous study [18] with slight modification which was further explained at the materials and methods sections in supplementary file.

Scanning Electron Microscopy (SEM) and Fourier transform-infrared (FT-IR) spectroscopy analysis

The lyophilized BNC samples were analyzed by SEM and FT-IR by following previous study [18] with slight modification which was further explained at the materials and methods sections in supplementary file.

X-ray-diffractometer (XRD) analysis

To investigate the crystallinity of the lyophilized BNC, X-ray diffractometer (D/Max-2500, Rigaku, Japan) analysis was performed using a cooper X-ray source. The diffracted radiation was measured in the range 2θ = 5° to 50°, and the crystallinity index of cellulose, CrI, was calculated through the following equation [19]:

$$CrI\ (\%) = [(I_{200}-I_{am})/I_{200}] \times 100,$$

where I_{200} represents the total intensity (i.e., crystalline region) at approximately $2\theta = 22.7°$ and I_{am} represents the baseline intensity (i.e., amorphous region) at approximately $2\theta = 18°$.

Analysis of protein binding to the crystalline region of cellulose

Quantitative analysis of the surface crystallinity of BNC was indirectly measured using *Ct*CBD3 (UniProtKB: Q06851), a cellulose-binding protein derived from *Clostridium thermocellum* (ATCC 27405), by following previous study [20] with slight modification which was further explained at the materials and methods sections in supplementary file.

Results And Discussion

Comparison of BNC production

At first, the effect of the different cellulosic additives upon BNC production by *Komagataeibacter* sp. SFCB22-18 was investigated. As cellulosic additives, Avicel (microcrystalline cellulose produced by acid hydrolysis of wood pulp) and carboxymethylcellulose (CMC; cellulose derivatives with carboxymethyl groups at some of the hydroxyl groups of glucopyranose) were used. Since these cellulosic additives have been widely used in the food, pharmaceutical, paper, and cosmetic industries due to their unique physical and chemical properties [21, 22], they are good candidate of altering BNC properties [23, 24]. In comparison with the control group (i.e., without additives, 0.4 g/L), Avicel and CMC addition showed slight increase in the BNC production (Fig. 1). With the additions of 0.1% Avicel and CMC, the *Komagataeibacter* strain produced 0.5 g/L and 0.7 g/L of cellulose pellicles, respectively. As the concentrations of cellulosic additives increased to 1%, BNC production also increased considerably. In particular, the addition of 1% CMC showed the highest BNC production of 2.0 g/L, which is equivalent to 5 times higher than in the control group. This increase might be because of the proper incorporation (or adsorption) of additives and reduction in crystallinity, which is a rate limiting step during BNC production [23, 25]. Meanwhile, Avicel did not show much improvement in BNC production because Avicel is less soluble than CMC [26]. Without optimization of reaction conditions, it was again proved that soluble additives may give more beneficial effects on production of BNC [23, 24, 27].

Morphological and structural properties of modified-BNC

The morphological structures of BNC with and without cellulosic additives were analyzed by SEM (Fig. 2). In the pure BNC, the thread-like parallel stacked cellulose bundles with large clumps by aggregation were observed. However, the clumps were not observable in altered BNC, possibly attributed by adsorption of additives on BCN surfaces [28–30]. This is probably due to the limitations of intermolecular interactions among cellulose fibrils, such as hydrogen bonding, the van der Waals force, and the electrostatic interactions which stabilize the highly ordered BC structure [14]. Accordingly CMC-altered BNC consisted of fibers having slightly further distance than Avicel-altered BNC, because of repulsive force. Therefore, cellulosic additives were efficiently incorporated into BNC, thereby modifying its structural morphology and crystalline properties.

The FT-IR spectra (Supplementary Fig. 1) of pure BNC and altered BNC were showing a broad OH peak stretching in the range of 3,500-3,000 cm^{-1} and C-O-C stretching at around 1,160 cm^{-1}, typical BNC spectrum reported [31, 32]. In pure BNC, additional peaks of 1,160 cm^{-1} (C-O-C stretching) and 1,035–

1,060 cm^{-1} (C-O stretching) were observed [31, 32]. In Avicel- and CMC-altered BNC, the peak intensity at 3,500-3,000 cm^{-1} increased in comparison with that in pure BNC. In addition, at Avicel-altered BNC, the peak intensities at around 1,620–1,650 cm^{-1} (OH bending) increased, showing stronger adsorption of water molecules to BNC. This may be due to either the inhibition of BNC crystallization by reducing the degree of polymerization (DP) of the modified BNC [33] or the exposure of a many OH groups by disruption of intermolecular bonds in BNC [23]. At CMC-altered BNC, strong absorption peaks at 1,572 cm^{-1}, corresponding to the carboxyl group [34] was observed, implying that CMC was well incorporated (or adsorbed) on pure BNC.

Degree of crystallinity

The crystallinity of cellulose may indirectly reflect its physical properties such as hardness, elasticity, permeability, and reactivity [35]. According to a previous report, BNC crystallinity is negatively related to polymerization of glucose [36]. Therefore, we analyzed and compared crystalline properties of BNC modified by the addition of cellulosic additives using XRD and crystalline cellulose-binding proteins.

At first, the crystallinity of BNC through the addition of different cellulosic substrates was measured by XRD (Supplementary Fig. 2). The XRD pattern of pure BNC showed strong diffraction peaks at $2\theta = 22.7°$ (principal peak), 14.5°, and 16.8°, representing cellulose Iα, which is naturally produced crystalline cellulose [37]. The Avicel- and CMC-altered BNC showed similar patterns to cellulose I. However, as the concentrations of additives increased, the diffraction peaks at the 2θ angles of 16.8° decreased considerably in comparison with pure BNC. This means that the cellulose I structure was transformed to cellulose allomorph, resulting in the conversion of some crystalline structures into amorphous structures through the addition of cellulosic substrates during BNC production [38, 39]. Furthermore, the crystallinity index of BNC measured by XRD was reduced by the addition of Avicel and CMC (Table 1); for example, while the crystallinity of pure BNC was 77.6%, those of modified BNC were about 69.2–73.4%. Similarly, when calculating the total crystallinity index (TCI, A1375/A2900 from FT-IR), a slight decrease in TCI values was observed after addition of cellulosic substrates. These observation agrees well with the previous studies indicating the cellulosic additives slightly hinder the crystallization of BNC during BNC synthesis, of which results are in accordance with the increase in BNC production or the increase in water retention ability (i.e. OH group exposure at FT-IR).

Table 1
Summary of the crystallinities of BNC incorporating different concentrations of cellulosic additives

		CrI (%)	**TCI (%)**	**Relative value of CBM binding (%)**
BC		77.6	0.994	100
Avicel		74.1	0.991	110.8
CMC		1.2	0.999	17.5
	Additive conc. (%)	CrI (%)	TCI (%)	Relative value of CBM binding (%)
BC + Avicel	0.1	72.2	0.993	243.3
	1.0	73.4	0.969	233.0
BC + CMC	0.1	69.2	0.988	177.8
	1.0	70.2	0.970	249.0

At second, Surface accessibility of the crystalline region of BNC was observed by using cellulose binding proteins. CBD has been suggested to understand the surface accessibility of cellulosic substrates [16, 40, 41] on the basis of the distinct molecular recognition ability whether the binding region in cellulose is crystalline or amorphous. It has not been previously reported that cellulose-binding proteins can be used to elucidate the morphology of the BNC surface, one of the nano-sized cellulosic materials. Among cellulose-binding proteins, we selected *Ct*CBD3, originating from *Clostridium thermocellum* ATCC 27405 and belongs to type-A cellulose-binding proteins, which predominantly bind to the crystalline region of cellulose. The amount of bound protein in pure BNC was approximately 1.9 nmol/mg substrate (Fig. 3). The modified-BNC samples showed 1.8–2.5 times higher binding affinities to *Ct*CBD3 than pure BNC. The differences to XRD results might be because cellulose binding protein are generally bound to the surface of the cellulose, while XRD or FT-IR focused on the fiber's interior structure and bulk properties [16]. Thus, the increase in protein binding in this study indicates that the crystalline regions of BNC were considerably exposed to the surface of modified BNC by cellulosic additives. This may suggest that the existence and stability of various functional groups were on the surface of modified-BNC. Therefore, the addition of cellulosic substrates such as Avicel and CMC during BNC synthesis can efficiently alter the surface crystallinity of the BNC during fermentation. In summary, since Avicel and CMC may attach to microfibrils surface during the production or crystallization of BNC, measuring binding properties using cellulose-binding protein may be a feasible option for identifying the crystalline properties of BNC [30, 33, 42].

Conclusions

In this study, cellulosic substrates with different crystalline properties, such as Avicel and CMC, were used to enhance BNC production and modify its surface crystallinity. Also, the surface-crystalline properties of BNC were compared using XRD and the cellulose-binding protein. The addition of cellulosic substrates improved the BNC production efficiently. Also, it was found that the cellulose-binding protein could become a more sensitive method than either FT-IR or XRD. The introduction of the binding protein may become a cost-effective and better alternative than the conventional method.

Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval, Consent to participate, and Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article and its additional information files.

Competing interests

The authors declare that they have no competing interests

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Figures

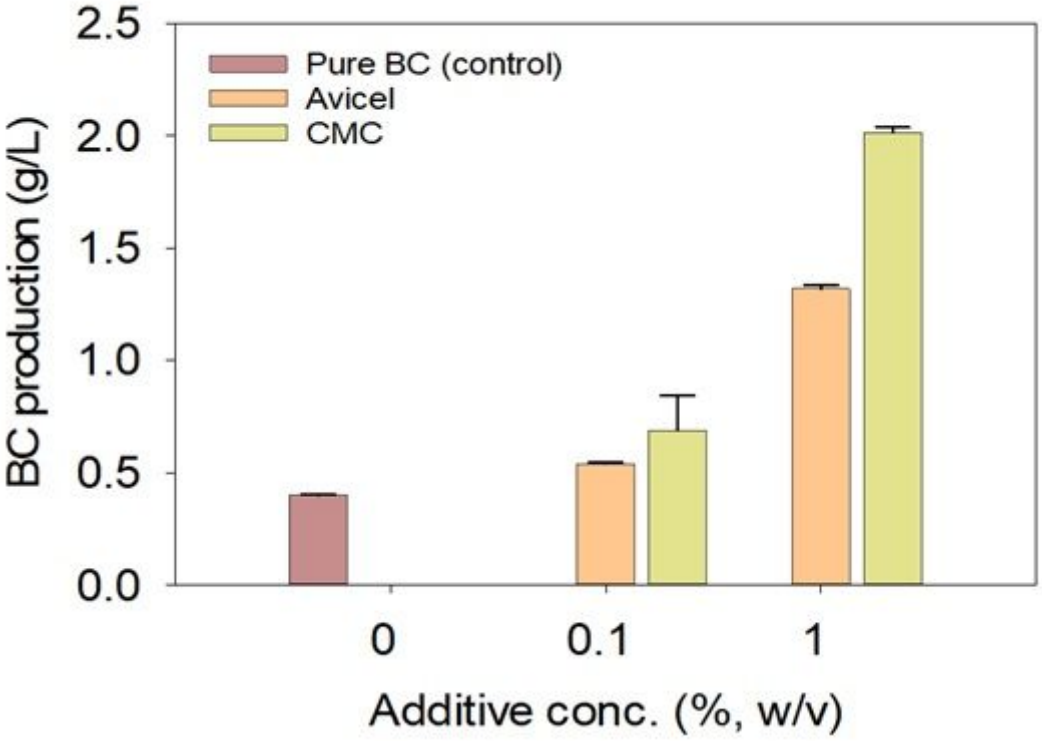

Figure 1

Production of bacterial cellulose by Komagataeibacter sp. SFCB22-18 in HSM medium containing different concentrations of Avicel and CMC. This experiment was performed in triplicate.

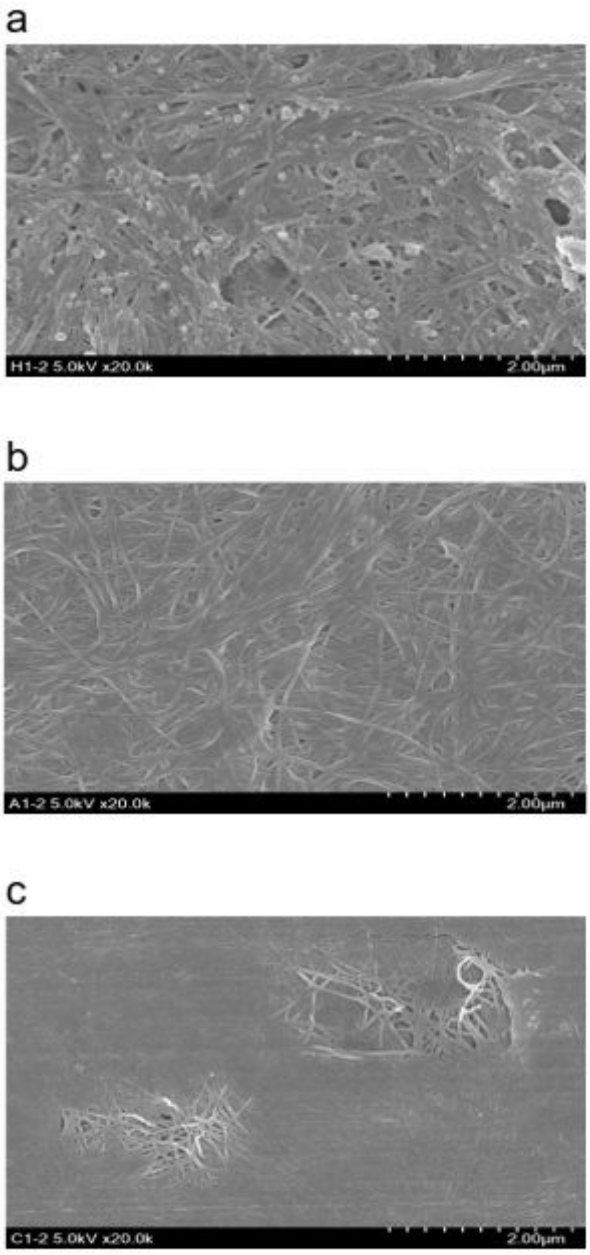

Figure 2

Scanning electron microscopy images of bacterial cellulose produced by Komagataeibacter sp. SFCB22-18 (a) without additives as a control; (b) with 1% (w/v) Avicel; and (c) with 1% (w/v) CMC.

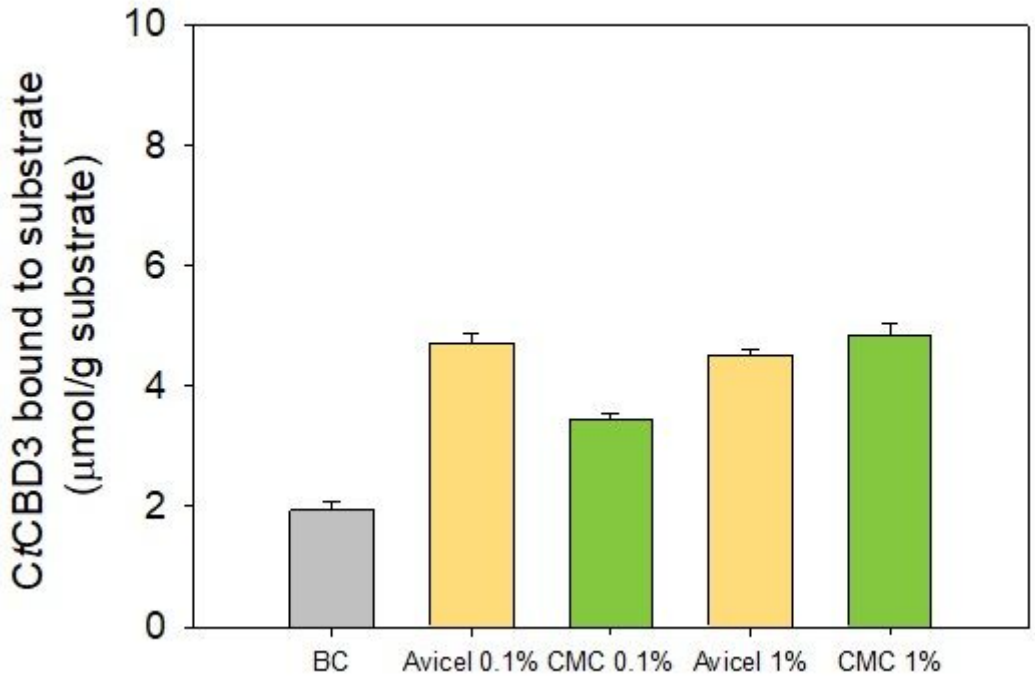

Figure 3

The binding ability of CtCBD3 to cellulose samples produced by Komagataeibacter sp. SFCB22-18 in an HSM medium containing different concentrations of Avicel and CMC. This experiment was performed in triplicate.

Supplementary Files

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