

# Cellulosic Nanomaterial Production Via Fermentation by *Komagataeibacter* sp. SFCB22-18 Isolated from Ripened Persimmons

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Bacterial nanocellulose (BNC) which is generally synthesized by several species of bacteria has a wide variety of industrial uses, particularly in the food and material industries. However, the low levels of BNC production during the fermentation process should be overcome to reduce its production cost. Therefore, in this study, we screened and identified a new cellulose-producing bacterium, optimized production of the cellulose, and investigated the morphological properties of the cellulosic materials. Out of 147 bacterial isolates from ripened fruits and traditional vinegars, strain SFCB22-18 showed the highest capacity for BNC production and was identified as *Komagataeibacter* sp. based on 16S rRNA sequence analysis. During 6-week fermentation of the strain using an optimized medium containing 3.0% glucose, 2.5% yeast extract, 0.24% acetic acid, 0.27% Na<sub>2</sub>HPO<sub>4</sub>, and 0.5% ethanol at 30°C, about 5 g/l of cellulosic material was produced. Both imaging and IR analysis proved that the produced cellulose would be nanoscale bacterial cellulose.

**Keywords:** Cellulosic nanomaterial, bacterial nanocellulose, *Komagataeibacter*, ripened persimmon, fermentation

## Introduction

Cellulose is one of the most abundant polymers in the world [1] and is composed of  $\beta$ -1,4-linked glucose units. It is generally synthesized by plants; however, bacteria are also capable of producing cellulose that is structurally similar to plant cellulose through fermentation [2]. This cellulose type is known as bacterial nanocellulose (BNC), which can be categorized as a cellulosic nanomaterial. It is produced by a number of bacterial species, including *Acetobacter*, *Alcaligenes*, *Azotobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella*, and *Sarcina* [3], which can be often observed during vinegar fermentation or fruit spoilage. However, the cultivation of cellulose-producing bacteria is inefficient since these bacteria require high oxygen levels and a long adaptation period, thereby resulting in high cost when produced on a commercial scale [4]. Hence, significant cost reduction by improvement of fermentation efficiency is required to make cellulose production a commercially viable option.

Until now, cellulose-producing bacteria have been isolated from various sources, such as fruits, flowers, fermented foods, and vegetable wastes [5–7]. However, the strategies like isolation and identification of new strains producing BNC as well as optimization of the fermentation process are still promising to obtain a high yield of BNC. Therefore, researchers have continued to investigate the isolation of microorganisms capable of greater BNC production. This goal is particularly vital since this nanomaterial has many possible applications arising from its diverse traits. For example, its gelation ability has made BNC a source of traditional foods like “Nata de coco” [8]. BNC is also expected to be an additive for use as a thickener, texturizer, and/or dietary fiber in food such as “Kombucha tea” [9, 10]. Besides the food industry, this cellulose nanomaterial can be used as a valuable material in the medical field in reconstructive surgery, in cell and tissue engineering as a drug carrier, and as electronic paper due to its high purity, high water content, high bio-compatibility, high mechanical strength, and gas permeability [6, 11–14]. In the present

study, the isolation and identification of cellulose-producing bacteria were performed from various sources including ripened fruits and traditional vinegars. Then, the fermentation conditions were optimized and the properties of the cellulose pellicles were identified.

## Materials and Methods

### Isolation and Screening of Cellulose-Producing Bacteria

To isolate bacteria that produce cellulose pellicles, we collected ripened fruits (citrus, persimmon, and tomato) and traditional vinegars made from apricot, banana, grape, lespedeza, peach, persimmon, Sanghwang mushroom, and rice. A piece of tissue from each fruit was dissected and placed in a 50-ml conical tube with 5 ml of distilled water. The tubes were vortexed and 100  $\mu$ l of the resultant liquid was spread on Hestrin-Schramm medium (HS medium; pH 6.0) containing 2.0% glucose, 0.5% yeast extract, 0.5% peptone, 0.27%  $\text{Na}_2\text{HPO}_4$ , 0.115% citric acid, and 2.0% agar [15]. Traditional vinegars (100  $\mu$ l) were diluted 1/5 and 1/10 and spread on HS plates. After incubation at 30°C for 5 days, individual isolates were transferred to new HS agar plates and further incubated at 30°C. The strains were also stored in 20% (w/w) glycerol at -80°C in the Seoul National University Fungal Collection (SFC, Korea) for further use.

### Selection and Identification of BNC-Producing Strains

To investigate BNC-producing ability, all strains isolated from ripened fruits and traditional vinegars were individually inoculated in 20 ml HS media. The formation of pellicles on the surface of the culture media was visually verified and the BNC-producing ability was compared after cultivation at 30°C for 7 days [16]. Then, the strain that produced the highest amount of cellulose was selected for further experiments.

The selected bacterial strain was identified based on 16S rRNA sequence analysis. Bacterial 16S rDNA was amplified using a Maxime PCR PreMix Kit (iNtRON Biotechnology, Korea) with 27F forward and 1492R reverse primers [17]. Colony PCR amplification was performed with a PCR mixture containing 1.0  $\mu$ l of each primer, 23.0  $\mu$ l sterilized distilled water, and one colony of a bacterial strain. PCR conditions were as follows: denaturation at 95°C for 10 min, 35 cycles of elongation at 95°C for 40 sec, at 55°C for 40 sec, and at 72°C for 60 sec, and final extension at 72°C for 5 min. PCR products were separated and visualized via 1% agarose gel electrophoresis, and purified using an Expin PCR Purification Kit (GeneAll, Korea). Sequencing was performed using an automated DNA analyzer system (PRISM 3730XL DNA Analyzer, Applied Biosystems, USA) from Macrogen (Korea). The sequences were checked and edited using MEGA v.5 [18]. Identification was conducted using both BLAST, against the EzBioCloud database [19], and phylogenetic analysis based on the neighbor-joining method with Kimura-2 parameter and 1,000

bootstrap replicates. The sequence was deposited into GenBank under accession number MH045739.

### Optimization of Culture Conditions

Growth of the selected strain was examined by inoculating the strain in HS medium in 250-ml flasks and incubating at 30°C for 7 days with shaking at 200 rpm. Culture viability was examined by plating daily on HS agar after vigorous shaking and filtering through sterile four-layered gauze to detach cells from the cellulose, and counting the number of colony forming units (CFU) of viable bacterial cells. For BNC production, a colony of the selected strain was inoculated in 100 ml HS medium in a 250-ml flask and incubated at 30°C for 3 days with shaking at 200 rpm. Five percent (v/v) of the culture broth supernatant was then inoculated in 100 ml of medium in 250-ml flasks for the optimization of culture conditions.

To optimize culture conditions, the effect of various temperatures (20°C, 25°C, 30°C, and 37°C) and agitation on BNC production for 7 days was compared. BNC amounts were also compared to that of *Komagataeibacter xylinus* (ATCC10245), which is a well-known cellulose-producing bacteria [20]. In addition, the composition of the growth medium was optimized by adding various carbon sources (glucose, mannitol, fructose, sucrose, maltose, and lactose; concentrations 1%–5%), nitrogen sources (yeast extract, corn steep liquor, beef extract, malt extract, and peptone; 0.5%–3%), acids (acetic acid, lactic acid, and succinic acid; 0%–0.3%), phosphate sources ( $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ , and  $(\text{NH}_4)_2\text{HPO}_4$ ; 0.27%) and ethanol 0%–2.5%.

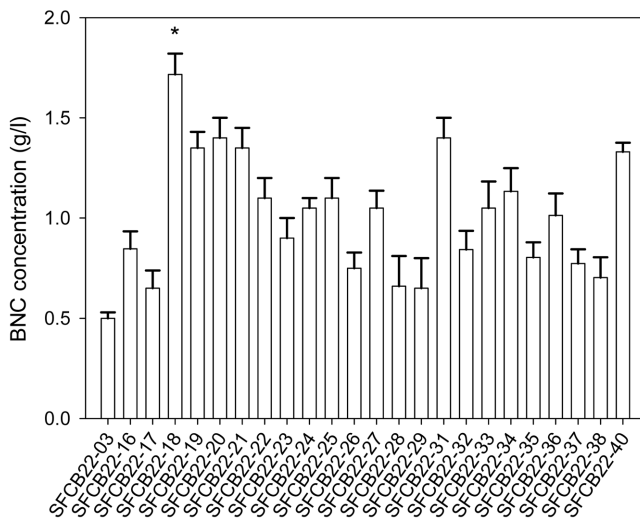
To determine statistical significance, a one-way analysis of variance with post hoc testing and a least significant difference test were performed using Statistica software (version 7.1, StatSoft, Tulsa, OK).

### Bacterial Nanocellulose Purification

BNC pellicles produced at the air-liquid interface of the medium were immersed in 0.3 N NaOH at 90°C for 20 min, placed on filter paper (No. 2; Advantec, Japan), and rinsed with distilled water until the pH of filtrate became neutral to ensure the complete removal of sodium hydroxide. The total solids content of the filtered pellicles was obtained by drying at 80°C for 48 h in triplicate.

### Analysis of BNC Properties by Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR)

To evaluate the conformational characteristics of the BNC fibrils obtained from the medium, the BNC pellicles were analyzed by FTIR (Nicolet iS5 FTIR Spectrometer, Thermo Scientific, USA) and SEM (SU8220; Hitachi, Japan). For FTIR analysis, the lyophilized sample was cut into pieces of a predetermined size and spectra in the range of 500 to 4,000  $\text{cm}^{-1}$  were obtained at a resolution of 4  $\text{cm}^{-1}$ , with 16 scans for each sample in the transmission mode. For SEM imaging, the lyophilized sample was coated with a thin platinum film and all images were taken at an accelerating voltage of 15 kV.



**Fig. 1.** Comparison of bacterial nanocellulose production by strains isolated from ripened persimmons. Box with asterisk indicates significantly different at  $p < 0.05$ .

## Results and Discussion

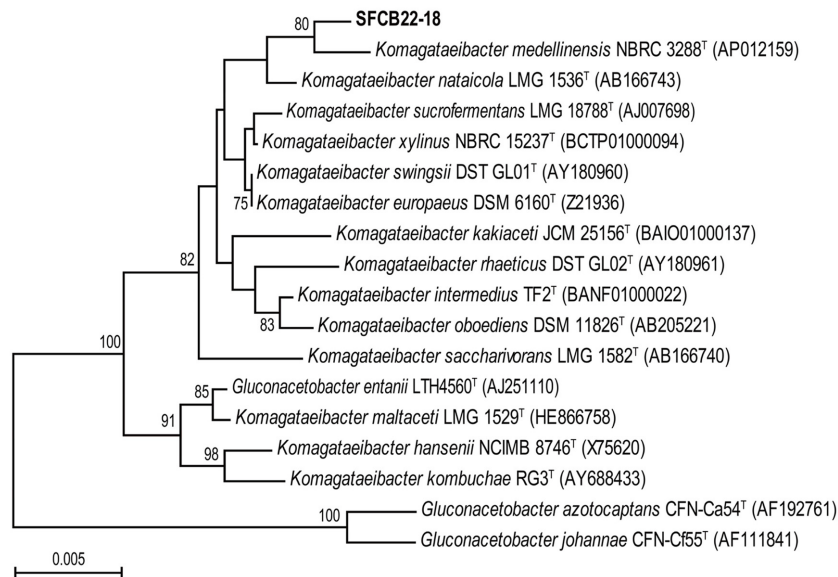
### Screening of Nanocellulose-Producing Bacteria and Identification of the Isolate

A total of 147 bacterial strains were isolated from various fruits (citrus fruits-11 strains, persimmons-92, and tomatoes-9) and traditional vinegars (apricot-4, banana-1, Chinese matrimony vine-2, lespedeza-10, peach-3, persimmon-6,

Sanghwang mushroom-2, and rice-7) using HS medium. Among them, 24 strains isolated from ripened persimmons only produced extracellular compounds, which could be cellulose polymer. Particularly, SFCB22-18 produced the greatest amount of polymer (1.70 g/l) (Fig. 1).

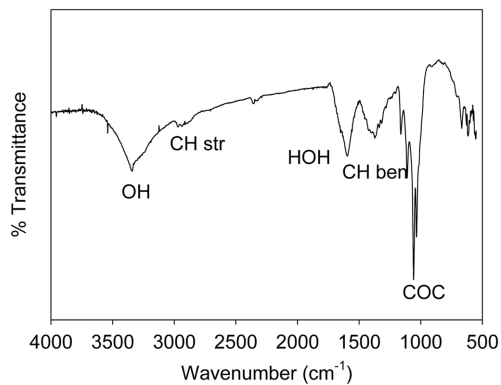
Strain SFCB22-18 was identified based on its 16S rRNA sequence. Primary identification using BLAST against the EzBioCloud database showed similarities of 99.7%, 99.6%, and 99.6% to *K. intermedius* TF2<sup>T</sup>, *K. oboediens* DSM 11826<sup>T</sup>, and *K. medellinensis* NBRC 3288<sup>T</sup>, respectively. Phylogenetic analysis based on the neighbor-joining method showed that strain SFCB22-18 was close to *K. medellinensis* NBRC 3288<sup>T</sup> with a high support value (80%), while it was clustered with several *Komagataeibacter* spp. (Fig. 2). Thus, the isolated strain remained as *Komagataeibacter* sp. SFCB22-18 because of its unclear phylogenetic relationship with closely related species.

So far, either acetic acid-producing species or polysaccharide-producing species have been isolated from persimmons and persimmon vinegars. For example, *Acetobacter syzygii*, *K. intermedius* (= *Gluconacetobacter intermedius*) and *K. europaeus* were observed during the acetification of persimmon [21]. In addition, *Gluconacetobacter* sp. RKY5, *K. xylinus* TJU-S8, *K. xylinus* KJ-1 (= *A. xylinum*), and *K. intermedius* TF2 were observed in persimmon vinegars [22, 23]. Mostly, they are producing insoluble cellulose polymers and soluble extracellular polysaccharides. In this study, we have found that some of the bacterial stains



**Fig. 2.** Neighbor-joining tree inferred from 16S rRNA showing the relationship of strain SFCB22-18 with the most closely related members of the genus *Komagataeibacter*.

Bootstrap scores of >70 are presented at the nodes. The scale bar indicates the number of nucleotide substitutions per site.



**Fig. 3.** FTIR spectra of bacterial nanocellulose.

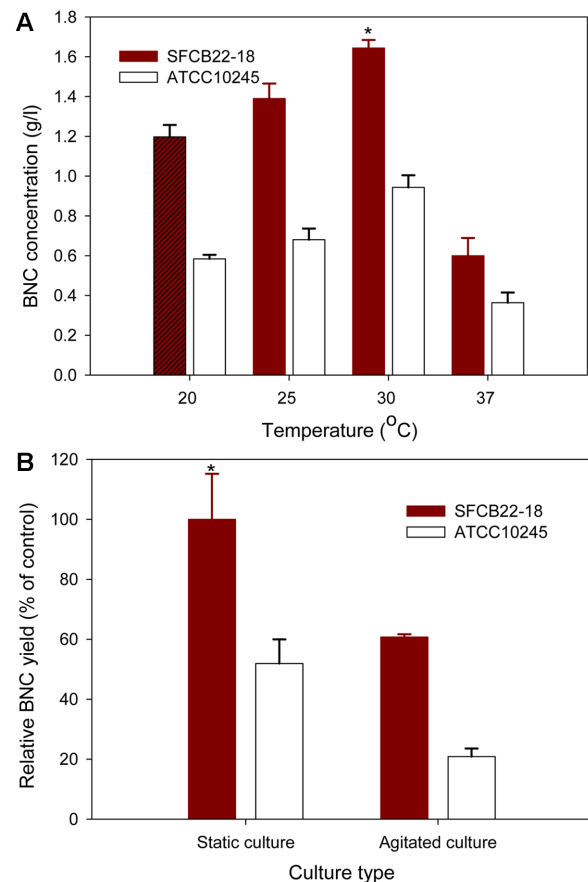
OH: O-H stretching, CH str: C-H stretching, HOH: H-O-H stretching, CH ben: symmetric deformation and bending vibration of C-H, COC: C-O-C stretching

isolated from ripened persimmons were able to produce insoluble extracellular polysaccharide. The extracellular polysaccharide which would be assumed as cellulose was examined with FTIR (Fig. 3). A typical absorption spectrum of BNC was observed, such as OH stretching at  $3,400\text{ cm}^{-1}$ , CH stretching at  $2,897\text{ cm}^{-1}$ , COC stretching at  $1,030\text{ cm}^{-1}$ , etc. [24, 25]. Thus, we assumed that the produced extracellular pellicles from *Komagataeibacter* sp. SFCB22-18 are mainly composed of cellulose.

### Optimization of Culture Parameters

BNC is a major form of exopolysaccharide that is produced on the surface of bacterial cultures in a slow fermentation process. In addition, BNC production is dependent on the strain type and on favorable cultivation conditions [26]. Thus, in this study, the growth and cellulose production in *Komagataeibacter* sp. SFCB22-18 under various fermentation conditions were investigated. The growth curve of *Komagataeibacter* sp. SFCB22-18 cultivated on HS media at  $30\text{ }^{\circ}\text{C}$  showed a steep increase to  $3.8 \times 10^6$  CFU/ml in 3 days. Since there was no further significant increase in cell growth during the 7 days of cultivation, the inoculums cultured for 3 days were used to optimize the batch fermentation conditions, including medium components, on BNC production.

Initially, the effects of fermentation temperature and agitation on BNC production were investigated (Fig. 4). As the culture temperature increased from  $20\text{ }^{\circ}\text{C}$  to  $30\text{ }^{\circ}\text{C}$ , BNC concentration after 7 days, fermentation increased from  $1.2\text{ g/l}$  to  $1.6\text{ g/l}$  (Fig. 4A). However, a further increase in temperature to  $37\text{ }^{\circ}\text{C}$  reduced BNC production, which is similar to previous results for BNC production in *K. xylinus*

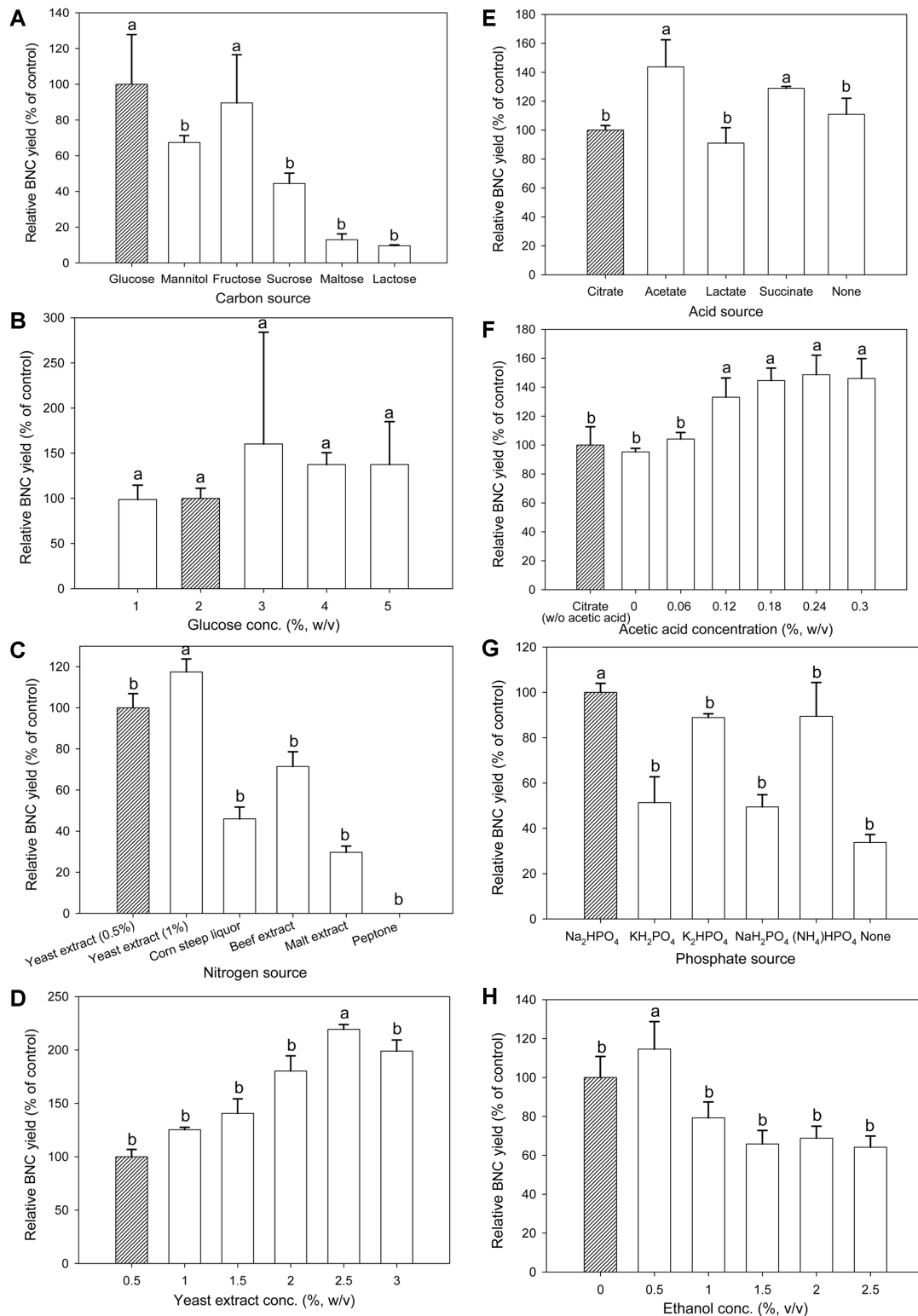


**Fig. 4.** Effects of (A) cultivation temperature and (B) agitation on the concentration of bacterial nanocellulose produced by *Komagataeibacter* sp. SFCB22-18.

Box with asterisk indicates significantly different at  $p < 0.05$ .

0416 and *Komagataeibacter* sp. PAP1 [27, 28]. The fermentation temperature of  $30\text{ }^{\circ}\text{C}$  was found to be optimal for efficient cellulose production in *Komagataeibacter* sp. SFCB22-18. Meanwhile, agitation during cultivation also affected the quantity and quality of the synthesized cellulose. When *Komagataeibacter* sp. SFCB22-18 was cultured with vigorous shaking at 200 rpm, BNC production significantly decreased by approximately 40% (Fig. 4B). These results suggested that agitation is not necessary to obtain a high amount of cellulose, probably due to a limitation in binding between newly synthesized BNC and existing BNC since cells might not have had enough time to anchor BNC at such high agitations [29]. Furthermore, a higher amount of cellulose was produced by *Komagataeibacter* sp. SFCB22-18 than the reference strain *K. xylinus* (ATCC10245). As a result, static culture condition was chosen for further experiments.

Next, the effects of different sources of carbon and nitrogen and their concentrations on BNC production were



**Fig. 5.** Effects of (A) carbon source, (B) glucose concentration, (C) nitrogen source, (D) yeast extract concentration, (E) acid source, (F) acid concentration, (G) phosphate source, and (H) ethanol concentration on the concentration of bacterial nanocellulose (BNC) produced by *Komagataeibacter* sp. SFCB22-18.

Box with patterns refers to the condition of Hestrin-Schramm (HS) medium. Bars with different letters are significantly different at  $p < 0.05$ .

investigated (Figs. 5A–5D). Among the various types of carbon sources used (glucose, fructose, lactose, maltose, mannitol, and sucrose), glucose addition (2% w/v, standard glucose concentration in HS media) led to the highest production of BNC, followed by the addition of the same concentrations of fructose and mannitol (Fig. 5A). According to previous studies, the optimal carbon source depends on the bacterial strain since bacteria have diverse metabolic activities. For example, *K. medellinensis* preferred glucose over sucrose and fructose, however, the amount of cellulose produced by *G. xylinus* was higher in the presence of fructose or glycerol than in the presence of glucose [30, 31]. When the glucose concentration increased to 3% (w/v), BNC production improved by 160% (Fig. 5B). When glucose is readily available, bacteria do not need to convert accessible carbohydrate sources into glucose molecules before polymerization into BNC, a process that uses energy. Thus, it is likely that glucose addition to the culture medium may lead to a greater production of cellulose. However, a further increase in glucose concentration to 5% did not show any significant increase in BNC production. Thus, a glucose concentration of 3% (w/v) was selected as the optimal carbon source for efficient BNC production by *Komagataeibacter* sp. SFCB22-18.

Among the various nitrogen sources used (yeast extract, corn steep liquor, beef extract, malt extract, and peptone), yeast extract (1% addition) led to the highest BNC production (approximate increase of 120% over BNC production in HS media) (Fig. 5C), which is in good agreement with results from previous studies for BNC production in *Acetobacter* sp. V6 and *A. lovaniensis* HBB5 [32, 33]. As the yeast extract concentration in the culture media increased to 2.5%, the amount of BNC produced increased to around 220% of that of the 0.5% yeast extract found in typical HS medium (Fig. 5D). Since further additions of yeast extract (3.0%) did not show beneficial effects in BNC production, 2.5% was chosen as the optimal yeast extract concentration for efficient cellulose production by *Komagataeibacter* sp. SFCB22-18.

Third, the effects of different sources of acid and the concentrations on BNC production were investigated (Figs. 5E and 5F). Various acid sources such as citrate, acetate, lactate, and succinate at concentrations of 0.115% were used individually to maintain the pH of the modified HS medium. Among them, the acetate-containing medium showed the highest BNC production, which was approximately 140% of that in standard HS medium (Fig. 5E). It is probably attributable to the higher pKa value of acetate than the other acid sources; thereby indicating that acetate

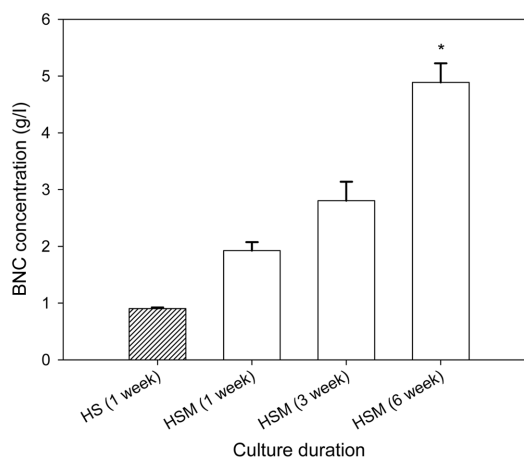
is the least toxic to cells during fermentation at pH 6.0. As the acetate concentration increased from 0–0.24%, the concentration of BNC pellicles continued to increase up to 149% of that produced in standard HS medium containing 0.115% citrate (Fig. 5F). Thus, 0.24% acetate concentration was selected as optimal for efficient BNC production by *Komagataeibacter* sp. SFCB22-18.

Fourth, the effects of the different sources of phosphate and the concentrations on BNC production were investigated (Fig. 5G). HS medium containing  $\text{Na}_2\text{HPO}_4$  led to the greatest production of BNC. HS media supplemented with  $\text{K}_2\text{HPO}_4$  and  $(\text{NH}_4)_2\text{HPO}_4$  also showed comparable BNC concentration. However,  $\text{KH}_2\text{PO}_4$  and  $\text{NaH}_2\text{PO}_4$  inhibited BNC production. This may be due to the fact that  $\text{Na}_2\text{HPO}_4$  is more basic than  $\text{NaH}_2\text{PO}_4$ , which can result in greater buffering activity during fermentation.

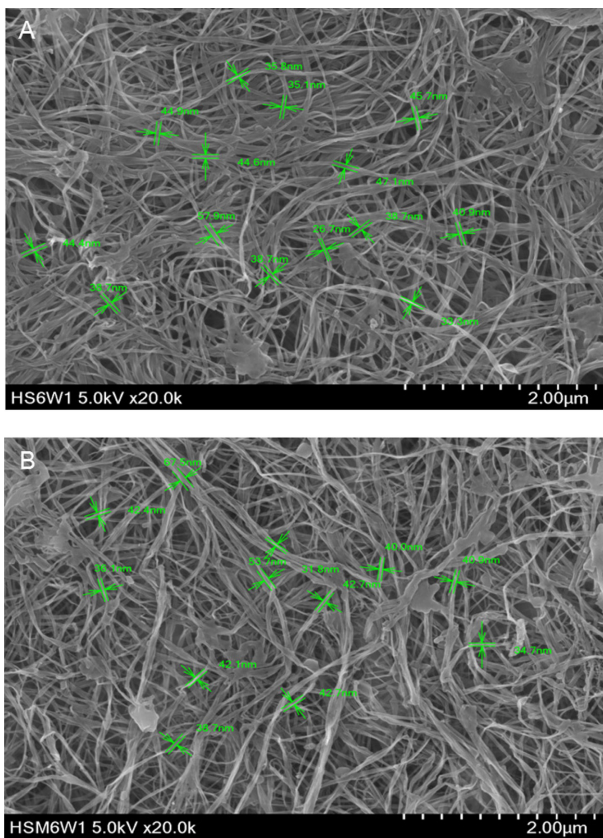
Finally, the effect of ethanol concentration was also investigated (Fig. 5H) because some acetic acid bacteria are capable of oxidizing ethanol [34], which might be used as an energy source. When 0.5% ethanol was added, BNC concentration in the medium after 7 days of cultivation increased 1.15 fold over production in HS medium without ethanol. However, further ethanol additions did not enhance BNC production probably because of the toxicity of ethanol and thus, 0.5% ethanol concentration was considered as optimal for efficient BNC production by *Komagataeibacter* sp. SFCB22-18.

### Time Profile of Bacterial Nanocellulose Production under Optimized Conditions

BNC production by the newly isolated *Komagataeibacter* sp. SFCB22-18 was explored under the optimized



**Fig. 6.** Bacterial nanocellulose concentration grown under optimized conditions during the six weeks of fermentation. Bars with asterisk indicates significantly different at  $p < 0.05$ .



**Fig. 7.** SEM images of bacterial nanocellulose synthesized by *Komagataeibacter* sp. SFCB22-18 after 6 weeks of cultivation in (A) typical Hestrin-Schramm (HS) medium and (B) modified medium (HSM).

conditions of 3.0% glucose, 2.5% yeast extract, 0.24% acetic acid, and 0.5% ethanol with 0.27%  $\text{Na}_2\text{HPO}_4$  as a phosphate source at 30°C without shaking. In comparison with BNC production in HS medium, *Komagataeibacter* sp. SFCB22-18 grown on the optimized medium produced about 2-fold higher BNC amounts (Fig. 6). In addition, BNC produced by *Komagataeibacter* sp. SFCB22-18 in the optimized medium increased continuously to up to 5 g/l during the six-week cultivation period, which is comparable to other studies showing 2–5 g/l by *Gluconacetobacter* sp. RKY5 and *K. sucrofermentans* DSM 15973 [22, 35, 36]. SEM images also showed that BC was composed of thread-like cellulose nanofibrils whose diameter was about 20–70 nm regardless of cultured medium according to random measurement of the fibrils (Fig. 7). Therefore, the strain, *Komagataeibacter* sp. SFCB22-18, has high potential for the production of bacterial nanocellulose.

In conclusion, we isolated an extracellular compound-producing strain, *Komagataeibacter* sp. SFCB22-18, from

ripened persimmons. The produced pellicles were characterized and identified with SEM and FTIR as nanocellulose. During the cultivation, approximately a 2-fold increase in BNC production was investigated using the optimized medium of 3.0% glucose, 2.5% yeast extract, 0.24% acetic acid, and 0.5% ethanol with 0.27%  $\text{Na}_2\text{HPO}_4$  at 30°C without shaking and 7 days of cultivation. The highest BNC concentration (4.9 g/l) was obtained after 6 weeks of cultivation. Therefore, the newly isolated strain, *Komagataeibacter* sp. SFCB22-18, was identified as a good producer of BNC which can be used in the food and material industries.

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### Conflict of Interest

The authors have no financial conflicts of interest to declare.

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