CELLULOSE NANOCOMPOSITES PRODUCTION THROUGH CO-CULTURE FERMENTATION

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by
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Abstract

Bacterial cellulose (BC) is a water-insoluble polysaccharide which consists of β-linked 1, 4 glucose residues. It exhibits several advantageous properties such as high purity, high crystallinity, a high degree of polymerization, high water content, and excellent biocompatibility comparing to other natural or regenerated cellulose. The extensively studied strains in the lab are *Gluconacetobacter xylinus* and *Gluconacetobacter hansenii* due to their relatively high yield of BC. Under static culture, cellulose in the pellicle form is synthesized by these strains at the air-liquid interface. The physiological structures behind the BC production are the linearly arranged terminal complexes (TCs) that distributed along the longitudinal surface of the strains. Glucan chains are secreted into the culture medium through the TCs while coupling with the crystallization process. Meanwhile, higher order cellulose fibrils are self-assembled through the process of co-crystallization, physical aggregation, and bundling. The presence of other materials in the culture medium such as exopolysaccharides (EPS), proteins, or inorganic chemicals could affect the crystallization or self-assembly process and result in the modifications of BC microstructure including the crystallinity, crystalline polymorphism, crystallite size, and ribbon width or endow new properties to BC.

Therefore, this study was focused on achieving the modifications on BC through co-culturing *G. hansenii* with *Escherichia coli* or *Lactococcus lactis*, both of which were able to secrete EPS. The BC-based nanocomposites were directly produced through co-culturing without the need of supplying additives into the culture media or further
modification processes. The effects of EPS on the assembly processes of BC were also explored.

The EPS extracted from *E. coli* ATCC 35860 under agitation conditions were chosen to study. After harvesting and purifying the EPS, its composition was analyzed by gas chromatography-mass (GC-MS) spectrometry. When adding the purified EPS into the culture media, another kind of EPS, composed of fructose, was produced by *G. xylinus* ATCC 23769 and a minor portion of the added EPS was incorporated into cellulose fibrillar network. The characteristics of BC nanocomposites synthesized in the presence of purified EPS was systematically studied through tensile testing, x-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and field emission scanning electron microscopy (FESEM). The results revealed that the EPS affected the cellulose-cellulose interactions during the physical aggregation of crystal microfibrils, but did not impact the co-crystallization process during BC synthesis. The addition of 4 mg/L or 8 mg/L purified EPS into the culture media, was found to significantly improve the tensile strength of BC nanocomposites while maintaining BC crystallinity and crystal size.

To simplify the production processes of BC pellicles with enhanced mechanical properties, a novel fermentation procedure, which is to co-culture *G. hansenii* with *E. coli* under static conditions, was developed. During co-culture, the mannose-rich EPS synthesized by *E. coli* were incorporated into the BC network and affected the aggregation of co-crystallized microfibrils without significantly changing the crystal sizes of BC. When co-culturing *G. hansenii* ATCC 23769 with *E. coli* ATCC 700728, which produced a low concentration of EPS at 3.3 ± 0.7 mg/L, but the BC pellicles exhibited a
Young’s modulus of 4,874 ± 1144 MPa and a stress at break of 80.7 ± 21.1 MPa, which are 81.9% and 79.3% higher than those of pure BC, respectively. The growth dynamics of the two co-cultured strains suggested that the production of EPS and BC were enhanced through co-culturing fermentation.

While co-culturing under static can manufacture BC-based nanocomposites, the incorporated amounts of EPS is difficult to adjust due to the static conditions. A novel two-vessel circulating system was developed in order to provide relatively static conditions for BC production while controlling the growth conditions for the co-cultured strain. *L. lactis* APJ3, which was genetically modified for the synthesis of hyaluronic acid (HA), was selected. The concentration of HA secreted by *L. lactis* APJ3 was controlled by adjusting the constant feed rate of glucose. The dynamic growth of the two strains revealed that *L. lactis* APJ3 was mainly growing within the first 48 hours while *G. hansenii* ATCC 23769 became active after 48 hours. The FTIR-ATR spectroscopy proved the incorporation of HA into the cellulose network. XRD analysis indicated that the presence of HA would not affect the crystallinity of BC/HA but increase the crystalline sizes. The FESEM images showed that more ribbons within the range of 20 – 40 nm diameter and larger ribbons between the range of 180 – 360 nm diameter were observed in BC/HA composites. The strain at break and the water holding capacity of BC/HA increased with the concentration of HA. The designed two-vessel circulating system provided a new method to directly produce BC-based nanocomposites.

Since the production of HA by *L. lactis* APJ3 was genetically engineered based on the P170 expression system, and the P170 promoter would be up-regulated as lactic acid accumulated, controlling the pH values of culture media was a straightforward
strategy to achieve the regulation on the yield of HA. The BC/HA nanocomposites were synthesized by co-culturing *G. hansenii* ATCC 23769 with *L. lactis* APJ3 under static conditions with different initial pH values of culture media. The HA concentration produced by *L. lactis* and the dry weight of BC/HA during co-culture were regulated by the initial pH values of culture media. The incorporation of HA into the cellulose network increased the crystal sizes when the initial pH values were at 7.0, 6.2, and 5.5. The strain at break was also increased while Young’s modulus was decreased when comparing BC/HA to pure BC produced under the initial pH values of culture media at 7.0 and 6.2. When the initial pH value was 4.0, the HA concentration in the culture media exhibited the lowest level observed, which was 20.4 ± 2.3 mg/L. The BC/HA composite synthesized under this condition exhibited an improved Young’s modulus of 5029 ± 1743 MPa from 2705 ± 656 MPa associated with the pure BC. The FESEM images showed that the presence of HA dramatically changed the distribution of ribbon width in BC/HA compared to that of pure BC.
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Chapter 1

Literature review

Bacterial cellulose (BC) has been widely used due to its unique properties. However, it also has some drawbacks such as high cost of production, poor biodegradability in the human body, and poor durability, especially in a moist environment. Researchers have tried to further the application of BC by producing BC-based nanocomposites (Hu, Chen, Yang, Li, & Wang, 2014; Huang et al., 2014). One of the methods was to supply additives during BC fermentation. For example, the effects of pectin, carboxymethyl cellulose (CMC), and gelatin on BC properties had been studied (Cheng, Catchmark, & Demirci, 2011; Gu & Catchmark, 2014). However, when it comes to large-scale production, using additives would further increase the production cost of BC. Therefore, co-culturing *Gluconacetobacter hansenii* with other strains that produce unique polysaccharides or proteins is an alternative option. Two kinds of polysaccharides, mannose-based EPS and hyaluronic acid that could be produced by other strains during co-culture are introduced.

1.1 Bacterial cellulose

Cellulose is the most abundant macromolecule on earth, and most of the cellulose is produced by vascular plants (R. M. Brown, 2004). However, there are also several microorganisms that synthesize cellulose including species of *Achromobacter*, *Alcaligenes*, *Aerobacter*, *Agrobacterium*, *Azotobacter*, *Gluconacetobacter*,
Pseudomonas, Rhizobium, Sarcina, Dickeya and Rhodobacter (Lin et al., 2013; Morgan, Strumillo, & Zimmer, 2013). BC was first reported by Adrian Brown while working with Bacterium aceti (A. J. Brown, 1886). He observed that sometimes a solid mass would form at the surface of the fermentation medium, which was later identified as cellulose. BC is a homopolymer consisting of β-linked 1, 4 glucose residues. Unlike plant cellulose, BC does not require extra processing to remove unwanted impurities such as lignin, hemicellulose, and pectin and therefore can retain a higher degree of polymerization (Yamanaka et al., 1989). Besides, BC sheets exhibit stronger tensile strength than plant cellulose due to its high purity (Tajima, Fujiwara, Takai, & Hayashi, 1995).

1.1.1 Structure of bacterial cellulose

BC produced from microorganisms has a unique mechanism in the synthesis of glucan chain molecules followed by a subtle self-assembly progress (Figure 1.1). Micrographs of the surface of the cell envelope indicate the presence of some 50-80 pore-like sites arranged in a regular row along the longitude of the cell (Moon, Martini, Nairn, Simonsen, & Youngblood, 2011). These pore-like sites are named terminal complexes (TCs) and each TC contains 3 subunits which are also linearly laid in the same direction as the TCs. In each subunit, there are at least 16 catalytic sites which form a matrix, and one catalytic site can produce one glucan chain through the polymerization of UDP-glucose units. These glucan chains are secreted into the culture medium while coupling with the crystallization process to form the subfibrils with a width of 1.5 nm. These subfibrils are then self-assembled to constitute microfibrils. Moreover, a fibrillar ribbon
is formed through subsequent aggregation with a width ca. 50-80 nm (R. M. Brown, 1996; Huang et al., 2014).

When the cellulose is extruded from the living cell, it is not completely crystallized (Benziman, Haigler, Brown, White, & Cooper, 1980) and the arrangements of cellulose crystal dependent on environmental conditions. Cellulose crystal usually consists of crystalline regions and amorphous regions. There are four polymorphs of crystalline cellulose which are cellulose I, II, III, and IV (Moon, Martini, Nairn, Simonsen, & Youngblood, 2011). Native cellulose is always cellulose I while the other three allomorphs are made by chemical or physical treatment of native cellulose (Gu, 2013). Cellulose I has two polymorphs, a triclinic structure (Iα) and a monoclinic structure (Iβ), which co-exist in various proportions depending on the cellulose source (Nishiyama, 2009). Generally, the cellulosics produced by primitive organisms including bacteria and algae is enriched in the cellulose Iα, whereas the cellulose from higher plant cell wall or in tunicates is mainly made up of cellulose Iβ (Belton, Tanner, Cartier, & Chanzy, 1989). The Iα and Iβ crystal structures are shown in Figure 1.2. The two allomorphs share nearly identical conformations, but different intermolecular hydrogen-bonding patterns. Within the hydrogen bonding plane, the number of the weak inter-chain hydrogen bonds in Iβ is believed to be greater than Iα which results in higher Iβ stability (Nishiyama, Sugiyama, Chanzy, & Langan, 2003).

The structure and ratio of crystalline and noncrystalline cellulose are essential for understanding the accessibility and reactivity of cellulose, as well as the properties of cellulose-based composites manufacturing (Gu, 2013). Crystallinity is defined as the
weight of crystalline portion of the cellulose divided by the total weight of crystalline and noncrystalline cellulose.

Figure 1.1 Schematic representation of the geometry of the terminal complexes (TCs) in relation to the size and shapes of microfibrils and ribbons synthesized by Gluconacetobacter strains (Acetobacter is the former name). The linear TC consists of 3 subunits and each subunit contains at least 16 catalytic sites (R. M. Brown, 1996).
Figure 1.2 Comparison of cellulose Iα and Iβ crystalline structure (Gu, 2013).

1.1.2 Pathways of bacterial cellulose synthesis

In recent years, various carbon sources including monosaccharides, oligosaccharides, alcohols, and organic acids, have been used to maximize bacterial cellulose production by various G. xylinus strains (the model strains) (Mikkelsen, Flanagan, Dykes, & Gidley, 2009). However, glucose-6-phosphate is the critical material which needs to be produced in order to synthesize bacterial cellulose, followed by isomerization of this intermediate to glucose-1-phosphate, catalyzed by phosphor-glucomutase, and conversion of the latter metabolite to UDP-glucose by UDPG-phrophosphorylase. Moreover, at last, UDP-glucose is catalyzed by cellulose synthase to form the β-(1, 4)-glucan chain (Figure 1.3). Besides, by-products through hexose monophosphate pathway and the tricarboxylic acid cycle would also consume glucose-6-phosphate to produce energy and materials for G. xylinus to grow (Ha, Shah, Ul-Islam, Khan, & Park, 2011).
1.1.3 Methods for producing BC based nanocomposites

Although the BC nanomaterial has unique physical and chemical characteristics, its high degree of crystallinity results in poor dissolubility and processability, thereby limiting the application of BC (Hu et al., 2014). However, BC possesses an abundance of hydroxyl groups on the surface, where modification can be easily achieved. The properties of BC derivatives are mainly determined by the type of functional groups. BC-based nanocomposites with specific properties have been created by changing functional groups or adding other surface functionalization using both biosynthetic or chemical modification approaches (Reiniati, Hrymak, & Margaritis, 2017).
1.1.3.1 Biosynthetic modification

The shape and structure of BC can be controlled by changing cultivation conditions such as the type of strains, carbon sources, incubation methods and additives (Heßler & Klemm, 2009; Dieter Klemm et al., 2006). The inclusion of additives in the nutrient media during biosynthesis can influence the assembly and microstructure of BC including the crystallinity, crystalline polymorphism, crystallite size, and ribbon width. Besides changing the structure of BC, through the inclusion of additives and dispersed particles, the generation of BC-based nanocomposites can also be effectively regulated during biosynthesis.

1.1.3.1.1 Altering BC structure

The pore system, elasticity of BC, water adsorption, and water holding capacity can be controlled through the incorporation of water-soluble polymers to the culture medium, such as carboxymethylcellulose (CMC), hydroxypropylmethyl cellulose (HPMC), methylcellulose (MC), poly (vinyl alcohol) (PVA) and polyethylene glycol (PEG). The addition of HPMC, CMC, and MC could cause decreased crystallinity and crystal size, as well as higher thermal stability and pore size (H.-H. Chen, Chen, Huang, & Lin, 2011). The presence of PVA in the culture medium resulted in a reduced water absorption ability and a slightly higher copper ion capacity compared with native BC. Moreover, the addition of PEG 400 caused a remarkable pore size increase (Heßler & Klemm, 2009).

1.1.3.1.2 Nanocomposites

Additives can also be incorporated into the growing BC fibrils to synthesize novel types of nanocomposites. It is a significant and specific modification method of BC.
Nowadays, various additives such as organic compounds, polymers, and inorganic substances have been applied to obtain BC based nanocomposites (D. Klemm et al., 2011).

For examples of organic compounds and polymers, the adding of poly(ethylene oxide) (E. E. Brown & Laborie, 2007), PVA (Gea, Bilotti, Reynolds, Soykeabkeaw, & Peijs, 2010) and starch (Grande et al., 2009) in the medium could incorporate into the network of BC to form BC-based nanocomposites. Along with the increasing of additive concentrations, the cellulose crystallized into smaller nanofibres, which further bonded together into bundles. The nanocomposites typically showed significantly improved mechanical properties, and the BC nanofibres were well dispersed in the composites.

The inorganic additives can also improve the performance of BC. In an agitated culture, BC-multiwalled carbon nanotubes (MWCNTs) composites would be obtained in the presence of MWCNTs (Park, Kim, Kwon, Hong, & Jin, 2009). In the static culture, band-like assemblies with sharp bends and rigidness were produced in the presence of MWCNTs. Simultaneously, the crystallinity index, crystallite size, and cellulose Iα content also changed, which might be attributed to the interaction between the hydroxyl groups of treated MWCNTs and the sub-elementary BC fibrils, interfering with the aggregation and crystallization of BC microfibrils. The SiO2 or TiO2 nanoparticles could also be incorporated onto BC microfibrils by adding silica or titanium precursor into the static medium (Geng et al., 2011). It was inferred that the basic proteins in the outer membrane of bacterium cell acted as the catalyst for the hydrolysis and condensation of the inorganic precursor, the surface of both outer membrane and BC nanofibres rendered the nucleation and growth sites for inorganic nanoparticles (Hu et al., 2014).
1.1.3.2 Chemical surface modification

Chemical modification usually has more clearly defined objectives compared to the biosynthesis method. Generally, the techniques are the same as those used in surface modification of wood products. BC can also be carboxymethylated, acetylated, phosphorylated, and modified by other graft copolymerization and crosslinking reaction to obtain a series of BC derivatives (Gandini & Belgacem, 2015). The introduction of new functional groups to the BC structure can endow BC with various features (Hu et al., 2014). For example, as cellulose has prodigious hydroxyl groups at the surface, techniques that react with alcohols, e.g., isocyanates, epoxides, acid halides, and acid anhydrides are the most common for direct attachment. These reactions could be used to form a host of other surface chemistries such as amine, ammonium, alkyl, hydroxyalkyl, ester (acetate and propionate), and acid. (Moon et al., 2011).

In most cases, the chemical modification of BC is focused on the improvement of its applicability and performance in different application fields. The functionalized diethylenetriamine-BC (EABC) (Shen et al., 2009), amidoximated BC (Am-BC) (S. Chen, Shen, Yu, Hu, & Wang, 2010), and phosphorylated BC (Oshima, Kondo, Ohto, Inoue, & Baba, 2008) were used as new adsorbents for metal ions. These novel adsorbents showed good adsorption performances for different metal ions and the microporous network structure of BC was maintained after the modification according to experimental data.

Besides, Berlioz, Molina-Boisseau, Nishiyama, and Heux (2009) reported that through vapor-phase technique, the surface esterification of BC microfibrils was achieved with the help of gaseous trifluoroacetic acid mixed with acetylating agents. Experimental
results had shown the acetylation proceeded from the surface to the interior crystalline core of BC nanofibers. Hence, for a moderate degree of substitution, the surface was fully grafted whereas the cellulose core remained unmodified and the original fibrous morphology was maintained.

1.1.4 Applications of BC and BC-based nanocomposites

Since BC and BC-based nanocomposites exhibit several unique properties, such as high tensile strength and water content, several recent publications have applied BC in various fields. The applications of BC and BC-based nanocomposites are summarized in the following areas.

1.1.4.1 Medical field

Winter (1962) discovered that healing, and specifically re-epithelialization, was accelerated if the wound was kept moist. The goals of wound healing are to recover the biological and structural function of the skin and to block scar tissue formations. Because of its unique properties, BC seems to be a suitable substitute for human skin to create a protective barrier as well as to deliver therapeutic compounds during the wound-healing. Thus, BC nanofibers were used as a template for the precipitation of silver (Ag) nanoparticles via in situ liquid phase redox reaction between AgNO₃ and NaBH₄. Ag nanoparticles with an average diameter of 1.5 nm were homogeneously spread on BC. Good dispersion and nano-dimension provide Ag particles a remarkably developed specific surface and consequently guarantees the hybrid nanofibers an efficient antimicrobial property. The colony-counting method was conducted to investigate the
antimicrobial activity against *E. coli* and the reduction in bacteria for the Ag/BC nanofibers was 99.2% (Yang, Yu, Sun, & Yang, 2011).

BC pellicle could also be used as a substrate for mammalian cell culture. Svensson et al. (2005) reported that the growth of bovine chondrocytes on BC pellicle exhibited that this BC pellicle supported bovine chondrocyte proliferation at about 50% more than that observed from collagen. Besides, BC film can be applied as an anti-adhesion and anti-proliferative material. Extremina, Fonseca, Granja, and Fonseca (2010) stated that cellulose triacetate (CTA) membranes with antibiotic imipenem (IPM) entrapped (CTA-IPM) were developed. The bacterial adhesion tests showed a statistically significant decrease in the adhesion of *Staphylococcus epidermidis* to CTA-IPM compared with adhesion to CTA alone.

### 1.1.4.2 Filtration

BC is attractive to be prepared as an adsorbing material for the remedy of pollution problems due to its advantageous properties: (1) high mechanical strength and excellent chemical stability, which are usually lacked in natural sorbents such as chitosan (Mladenova, Dakova, & Karadjova, 2011), and (2) ultrafine Nanofiber network and high specific surface area, which is closely related to adsorption efficiency. Oshima et al. (2008) prepared phosphorylated bacterial cellulose as an ion-exchanger that exhibited a high adsorption capacity for various transition metal ions and lanthanide ions.

### 1.1.4.3 Paper manufacturing

Johnson and Neogi (1989) reported that highly branched, reticulated BC pellets produced from agitation culture are suitable for the production of high-quality paper. The
fragments of bacterial cellulose were also found effective for reinforcing pulp papers and improved its folding endurance (Iguchi, Yamanaka, & Budhiono, 2000).

### 1.1.4.4 Acoustic transducer diaphragm

Yamanaka and Watanabe (1994) reported potential and possible applications of BC and its composites in the area of acoustic transducers. The exceptional shape retention ability of BC, measured as the Young’s Modulus, coupled with the high internal loss of the material made it ideal for speaker diaphragms. The novel diaphragms demonstrated two distinctive properties: high sonic velocity and low dynamic loss, and had been marketed by Sony Corp as loudspeakers and headphones (Iguchi et al., 2000).

### 1.1.4.5 Food application

The bacterial cellulose gel, named Nata (Phisalaphong & Chiaoprapobkij, 2012), originating from the Philippines, is a traditional dessert in Southeast Asia. There are many kinds of Nata, such as Nata de coco and Nata de pina, with their flavor being controlled by the culture medium resource: Nata de coco uses coconut while Nata de pina uses pineapple (Shi, Zhang, Phillips, & Yang, 2014).

Besides from raw materials for food, BC can also be used as excellent resources for food packing materials. Active food packaging generally either acts to increase or confirm the shelf life or safety of products. Packing material must be durable and retain barrier properties. To achieve those properties, Tomé et al. (2010) esterified a BC membrane and the product successfully showed better barrier properties towards vapor and gas, than the original BC membrane. Moreover, antimicrobial agents can also be incorporated into food packaging films to create an active packaging system which could maintain their activity during food storage. Jipa, Stoica-Guzun, and Stroescu (2012) used
BC containing Nisin to control *Listeria monocytogenes* and total aerobic bacteria on the surface of vacuum-packaged frankfurter sausages. According to their experiments, Nisin-containing BC films were effective in controlling *L. monocytogenes* and reducing total aerobic plate counts on the surface of frankfurter sausages.

### 1.2 Co-culture in fermentation

Co-culture has been wildly used in industrial biotechnology including the production of bulk chemicals, enzymes, food additives, antimicrobial substances, and microbial fuel cells. In co-cultures, degradation and metabolism of substrates occur by the combined metabolic activity of the known microbial strains under aseptic conditions resulting in the possibility of utilizing other cheaper substrates for industrial production. Cocultivation may also result in increased yields and product quality (Bader, Mast-Gerlach, Popovic, Bajpai, & Stahl, 2010).

#### 1.2.1 Research progress on co-culturing *G. hansenii* with other strains

Only a few publications about co-culturing *G. hansenii* with other strains have been published. Seto et al. (2006) cultured *G. xylinus* with *Lactobacillus mali* in corn steep liquor/sucrose liquid medium and observed that a threefold higher cellulose yield when compared to the *G. xylinus* monoculture. A similar enhancement was observed in co-culture with various *L. mali* strains but not with other *Lactobacillus* spp. The enhancement of cellulose production was most remarkable when sucrose was supplied as the substrate. *L. mali* mutants for exocellular polysaccharide (EPS) production were defective in promoting cellulose production, but the addition of EPS to the monoculture
of *G. xylinus* did not affect cellulose productivity. The author concluded that cell-cell interaction might assist by the EPS-producing *L. mali* promotes cellulose production in *G. xylinus* since scanning electron microscopic observation of the cocultures revealed a frequent association between the *G. xylinus* and *L. mali* cells.

Another example is the co-culture of *G. xylinus* with *Escherichia coli* (Qin, Panilaitis, & Kaplan, 2014). The compatibility of *G. xylinus* and recombinant *E. coli* strains was first investigated. However, in this example, *E. coli* was not used to improve the quality or the production of BC. Instead, BC was used as a chassis for entrapping the engineered *E. coli*, allowing it for the detection mechanism to be extremely sensitive and resulting in a significant fluorescent signal from a single receptor binding event.

Co-culturing *G. hansenii* with other strains which could produce unique polysaccharides is similar to applying additives during *G. hansenii* monoculture. The presence of polysaccharides in the media may interfere with *G. hansenii* or bind directly to the cellulose during production, thereby affecting the yield, structure, morphology and physical properties of BC.

### 1.2.2 Potential polysaccharides for co-culturing with *G. hansenii*

#### 1.2.2.1 Mannose-based EPS

Mannose-based EPS are known to exhibit a strong affinity to cellulose. Whitney, Brigham, Darke, Reid, and Gidley (1998) predicted strong interactions between mannose-based polysaccharides and cellulose due to the similarity of mannose to cellulose in stereochemistry with the only difference being the configuration of the hydroxyl group at the C2 position. In practice, during the pretreatment of lignocellulosic
materials, dissolved mannan could even be re-adsorbed onto the cellulose (Wang, Li, Yang, Wang, & Zhang, 2016).

1.2.2.2 Hyaluronic acid

Hyaluronic acid is a high-value polysaccharide which has been applied in the cosmetic, biomedical, and food industries. The structure of the repeating unit of HA is shown in Figure 1.4. The price for pure HA will be in the range of USD 10,000 - 100,000/kg based on the purity where the medical grade HA will be in the higher price end (Liu, Liu, Li, Du, & Chen, 2011). HA will be beneficial for the migration, proliferation, and differentiation of cells during the wound healing process (Y. Li, Qing, Zhou, & Yang, 2014). Ying Li et al. (2015) had produced HA/BC nanocomposite films through solution impregnation method and discovered that the HA/BC with 0.1% HA had the shortest wound healing time while HA/BC with 0.05% HA obtained the best tissue repair results comparing with the pure BC during in vivo experiments.

Figure 1.4 Structure of disaccharide repeating unit of HA (Liu et al., 2011).
1.3 References


Chapter 2

Research objectives

To modify the properties of BC and expand its applications, a wide variety of additives have been included in the culture medium aiming to achieve unique modification during the biosynthesis of BC. However, supplying purified additives will increase the already high production cost of BC. One alternative method that would achieve \textit{in-vivo} BC modification while not raising the production cost is by co-culture fermentation. The principal objective of this research is to produce BC nanocomposites through co-culturing a BC-producing strain with other microbes that could secrete unique polysaccharides. Specifically, \textit{Gluconacetobacter hansenii} ATCC 23769 was chosen for BC production since it would not produce significant amounts of exopolysaccharides (EPS) that may interfere with EPS from other co-cultured organisms. \textit{Escherichia coli} and \textit{Lactococcus Lactis} APJ3 were selected due to their secretion of mannose-based EPS and hyaluronic acid, respectively. Several hypotheses needed to be investigated during the research are as follows:

1. The mannose-based EPS produced by \textit{E. coli} would have an affinity to cellulose and result in the modification of BC which obtained enhanced mechanical properties (the affinity of hyaluronic acid to cellulose and its effects on BC synthesis have been reported).

2. \textit{G. hansenii} and \textit{E. coli/L. lactis} could be co-cultured in one culture medium to maintain their own growth and to produce bacterial cellulose nanocomposites.

3. With the presence of mannose-based EPS or hyaluronic acid produced by the co-cultured strain, the properties of bacterial cellulose could be modified through
changing the crystallization, bundling, or network formation of BC during the co-culture of *G. hansenii* and *E. coli*/*L. lactis*. The different concentrations of mannose-based EPS or hyaluronic acid produced by the co-cultured strain would have different effects on the properties of bacterial cellulose.

4. The concentration of hyaluronic acid could be adjusted by controlling the fermentation parameters while maintaining the production of bacterial cellulose nanocomposites.

Firstly, the research was to study the effects of mannose-based EPS produced by *E. coli* on the properties of bacterial cellulose (Chapter 3). The mannose-based EPS produced by *E. coli* was harvested, purified, and added to the culture medium with different concentrations at the beginning of BC fermentation. Based on the results from Chapter 3, two *E. coli* strains were selected where *E. coli* ATCC 700728 produced mannose-based EPS at 3.3 ± 0.7 mg/L and *E. coli* ATCC 35860 synthesized mannose-based EPS at 41.4 ± 3.8 mg/L under static conditions. The properties of BC nanocomposites produced by co-culturing *G. hansenii* and *E. coli* (ATCC 700728 or ATCC 35860) under static conditions were studied (Chapter 4). However, under static conditions, little control of fermentation parameters could be achieved. Moreover, the compositions of mannose-based EPS produced by *E. coli* were sensitive to the fermentation conditions. To achieve the control on EPS production during co-culture, the co-cultured strain was changed to *L. lactis* APJ3 which was genetically modified to produce HA. A novel designed two-vessel circulating system was also developed to replace the static fermentation (Chapter 5). Through control of constant feed rates of glucose, the HA concentration in the co-culture media was adjustable. The properties of
BC/HA produced under different constant feed rates of glucose were studied as well. Since the yield of HA by *L. lactis* was directly affected by the extracellular pH, an alternative method to control the HA concentration was proposed which was by varying initial pH values of culture media during co-culturing *G. hansenii* and *L. lactis* under static conditions (Chapter 6). Finally, a summary of the conclusions and recommendations for future research are presented (Chapter 7).
Chapter 3

Effects of exopolysaccharides from *Escherichia coli* ATCC 35860 on the mechanical properties of bacterial cellulose nanocomposites

3.1 Abstract

The effects of growing bacterial cellulose (BC) in the presence of exopolysaccharides (EPS) extracted from *Escherichia coli* ATCC 35860 on the mechanical properties of BC have been studied. After harvesting and purifying the EPS, its composition was analyzed by gas chromatography-mass spectrometry. When adding the purified EPS into the culture media, another kind of EPS, composed of fructose, was produced by *Gluconacetobacter xylinus* ATCC 23769 and a minor portion of the added EPS was incorporated into cellulose fibrillar network. The characteristics of BC nanocomposites synthesized in the presence of purified EPS was systematically studied through tensile testing, x-ray diffraction, Fourier transform infrared spectroscopy, and field emission scanning electron microscopy. The results revealed that the EPS affected the cellulose-cellulose interactions during the physical aggregation of crystal microfibrils, but did not impact the co-crystallization process during BC synthesis. The addition of 4 mg/L or 8 mg/L purified EPS into the culture media, was found to significantly improve the tensile strength of BC nanocomposites while maintaining BC crystallinity and crystal size.
3.2 Introduction

Bacterial cellulose (BC) is a water-insoluble polysaccharide produced by many types of microorganisms, especially *Gluconacetobacter xylinus* and *Gluconacetobacter hansenii*, which are widely used in laboratories due to their relatively high cellulose yield. Compared with other natural or artificially synthesized cellulose, BC exhibits several advantageous properties, such as high purity, high crystallinity (up to 90%), high degree of polymerization (up to 10,000), high water content (up to 99%), and excellent biological affinity (Barud et al., 2011; Hu, Chen, Yang, Li, & Wang, 2014; Reiniati, Hrymak, & Margaritis, 2017). These characteristics have resulted in BC being widely used in diverse fields including biomedical, water filtration, paper manufacturing, and food production (Huang et al., 2014; S. Lin et al., 2013). In order to expand the applications of BC, two strategies have been applied to modify its properties including *in situ* and *ex situ* methods (Stumpf, Yang, Zhang, & Cao, 2018). Typically, the *in situ* method is to modify BC during the growth of BC-producing strains by changing the culture conditions or adding additional materials into the culture media, whereas the *ex situ* modification is achieved after the BC has been harvested followed by chemical or physical treatments. Between the two methods, applying additional materials into culture media has been extensively studied by researchers aiming at modifying the microstructure of BC, including the crystallinity, crystalline polymorphism, crystallite size, ribbon width, and network formation or endowing new properties to BC, such as antibacterial, optical, mechanical, catalytic and biomedical functions (Dayal & Catchmark, 2016; Hu et al., 2014; S.-P. Lin et al., 2016).
Biofilms produced by many microorganisms are a matrix of EPS, proteins, lipids, and DNA, which could help the strains adhere to a variety of surfaces, provide protection from antimicrobials in the environment, and act as reservoirs for nutrients (Limoli, Jones, & Wozniak, 2015). These unique functions of biofilms are due to their diverse chemistries and distinct architectures that arise from the interaction between different EPS produced by the strains (Jenkinson & Lamont, 1997). The presence of EPS could affect the porosity, density, water content, sorption properties, and mechanical stability of biofilms (Flemming, Neu, & Wozniak, 2007). For *Escherichia coli*, its microcolony morphology depends on the production of amyloid curli fibers (composed of proteins) and exopolysaccharides, such as cellulose and colanic acid. These materials compose the biofilm, and the cellulose acts as an architectural element with a distinct spatial distribution within the system (Serra, Richter, & Hengge, 2013). Together with the other EPS and the curli fibers, a three-dimensional nanocomposite with tissue-like properties including stability, cohesion, and elasticity is formed, providing the function of cell adhesion and physical barrier formation. These properties make the embedded cells extremely hard to kill and result in more complex sterilization processes in water purification, biomedical, and food industries (Ryu & Beuchat, 2005). Although the formation of biofilms is not desirable in many fields, its robust structure and mechanical stability provide insights on how to further improve the mechanical properties of BC pellicles. Polysaccharides, including xanthan gum, chitin, pectin, agar, and hyaluronic acid, have been used as additives to study their effects on BC properties (Cheng, Catchmark, & Demirci, 2009; Li et al., 2015). However, studies on the effects of EPS
from biofilm-forming strains on BC properties have been rarely reported (Fang & Catchmark, 2014).

In this study, *Escherichia coli* ATCC 35860 was chosen, which is able to produce different kinds of EPS, such as colanic acid and polysialic acid, when changing culture conditions (Navasa, Rodriguez-Aparicio, Martinez-Blanco, Arcos, & Ferrero, 2009). With a specific culture condition (Hestrin-Schramm medium, 30°C, and 250 rpm) in this research, the EPS produced by *E. coli* ATCC 35860 was harvested, purified and analyzed by gas chromatography-mass (GC-MS) spectrometry. Different amounts of purified EPS were added to the culture media at the beginning of fermentation for the production of BC nanocomposites. The properties of these BC nanocomposites were systematically characterized by tensile testing, x-ray diffraction, Fourier transform infrared spectroscopy, and field emission scanning electron microscopy. The goal of this study was to evaluate the effects of the EPS from *E. coli* ATCC 35860 on the mechanical properties of bacterial cellulose and propose a possible mechanism responsible for the property changes.

### 3.3 Materials and methods

#### 3.3.1 Strains and culture conditions

*Gluconacetobacter hansenii* ATCC 23769 and *Escherichia coli* ATCC 35860 were purchased from the American Type Culture Collection (ATCC) and cultured statically and at 250 rpm respectively in the standard Hestrin-Schramm (HS) medium, containing 2% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.27% (w/v) sodium phosphate dibasic, 0.115% (w/v) citric acid, and 0.1% (w/v) magnesium sulfate.
(Hestrin & Schramm, 1954). The initial pH of HS media was adjusted to 5.0 by hydrochloric acid.

3.3.2 Purification and quantification of EPS from *E. coli* ATCC35860

EPS was harvested after culturing *E. coli* ATCC 35860 in HS media at 30°C and 250 rpm for 120 hours. The method used for EPS purification was that of Obadia et al. (2007). Briefly, the cell culture was heated to 100°C for 15 min to denature EPS-degrading enzymes. After cooling down, the culture samples were centrifuged at 13,200 \( \times g \) at 4°C for 30 min, and then the EPS in every 50 mL of supernatant was precipitated by adding three volumes of ethanol. The mixture was incubated at 4°C overnight and centrifuged at 13,200 \( \times g \) at 4°C for 30 min. The resulting pellet was dissolved in 5 mL distilled water, dialyzed with a 3.5 kDa molecular weight cutoff membrane (SnakeSkin Dialysis Tubing, Thermo Scientific) for 48 h against distilled water, and freeze-dried (model Freezoom 2.5 L, Labconco, Kansas, MO). Five milliliters of 10% (v/v) trichloroacetic acid (TCA) were used to re-dissolve the lyophilized powder and to precipitate residual polypeptides. The mixture was centrifuged again under the same condition as described above. The supernatant was dialyzed again for 5 days against distilled water and freeze-dried. The resulting powder was accurately weighed on an analytical balance (Mettler AE240, Greifensee, Switzerland) and stored at 4°C. Before the final freeze-drying, Bradford assay was applied to determine the protein concentration in dialyzed EPS solution to ensure the results were less than 1%.
3.3.3 Preparation of BC nanocomposites

To make the primary inoculum of *G. hansenii*, 100 µL of thawed frozen stock, 30 mL of HS media, and 100 µL of cellulase (Sigma) were shaken at 250 rpm and 30°C in a 100 mL Erlenmeyer glass flask for 1 to 2 days until an OD$_{600nm}$ reading of 0.9 was reached. The strains were centrifuged at 2,500 × g at 4°C for 5 min, washed three times with fresh HS media to remove the cellulase and re-suspended in fresh HS media with an OD$_{600nm}$ reading at 0.9. Different amounts of purified EPS, ranging from 4 mg/L, 8 mg/L, 40 mg/L, 200 mg/L to 1000 mg/L, were added to HS media with 1% (v/v) primary inoculum of *G. hansenii* to produce BC nanocomposites statically at 30°C. The BC produced with different amounts of EPS was named 4 mg/L EPS BC, 8 mg/L EPS BC, 40 mg/L EPS BC, 200 mg/L EPS BC and 1000 mg/L EPS BC, respectively. At the end of the 5-day incubation, the cellulose pellicles were collected and treated with 0.1 M NaOH solution at 80°C for 1 hour. The alkali treatment was repeated once followed by a deionized (DI) water rinse until a pH of 7.0 was achieved. The purified pellicles were lyophilized, accurately weighed, and stored in a desiccator for further analyses. The yield of cellulose was converted to g/L in dry weight. A control experiment without adding EPS was also performed simultaneously.

3.3.4 Monosaccharide analyses of EPS and BC nanocomposites

The monosaccharide analyses of EPS and BC nanocomposites were conducted by gas chromatography-mass spectrometry (GC-MS). The sample preparation was performed in two steps: (a) the hydrolysis of EPS and BC nanocomposites to obtain monosaccharides and (b) the trimethylsilyl (TMS) derivatization of monosaccharides.
For the hydrolysis of purified EPS, an aqueous solution of 2 M trifluoroacetic acid (TFA) was used resulting in a final concentration of EPS at 1 mg/mL. The mixture was sealed in glass vials and kept at 121°C for 2 h. To remove TFA, the resulting solution was evaporated under a stream of N₂ at 40°C. The dried hydrolysate was dissolved in DI water. The hydrolysis procedure of BC nanocomposites was described by Sluiter et al. (2008). Briefly, the BC nanocomposites were hydrolyzed in 72% H₂SO₄ at 30°C for 1 h, followed by diluting the concentration of H₂SO₄ to 4% with DI water and kept at 121°C for 1 h. The hydrolyzed solution was then neutralized with NaOH.

The TMS derivatization method was performed according to the procedures from Roessner, Wagner, Kopka, Trethewey, and Willmitzer (2000), with some modifications. Firstly, 20 µL of hydrolyzed samples were combined with 0.5 µL of inositol solution (1 mg/mL in 0.1% formic acid solution, Sigma) as the internal standard (IS). The mixture was freeze-dried, followed by adding 30 µL methoxyamine hydrochloride (MEOX) (20 mg/mL in pyridine, Sigma) and incubated on the heat block at 37°C for 1 h. 80 µL of N-methyl-N-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (MSTFA) (Sigma) was added, and the resulting mixed solution was incubated at 37°C for another hour in preparation for GC-MS analysis.

Monosaccharides were determined with an Agilent 7890A gas chromatography system coupled with an Agilent 5975 mass spectrometer (Agilent Technologies, Santa Clara, CA). A DB-5 (5%-Phenyl-methylpolysiloxane) capillary GC column (30 m × 0.25 mm I.D. × 0.25 µm film thickness, Agilent Technologies) was utilized with helium as the carrier gas at a constant flow rate of 1 mL/min. Samples were injected at a volume of 0.5 µL with a split ratio of 20:1. The total run time was 30 min, and the mass spectral data
were collected in full scan mode in a mass range of 30-300 m/z. In order to obtain the relative response factor (RRF) and calculate the molar percentage of each monosaccharide, the sugar standards (1 mg/mL, Sigma) were also tested under the same conditions described above.

3.3.5 Growth of *G. hansenii* ATCC 23769 in chemical media with EPS as the only carbon source

The chemical medium comprised with 1.0% (w/v) glucose, 0.3% (w/v) (NH₄)₂SO₄, 0.1% (w/v) KH₂PO₄, and 0.2% (w/v) Na₂HPO₄·12H₂O (pH 5.0) (Son et al., 2003), was used to test whether the purified EPS from *E. coli* ATCC 35860 could support the growth of *G. hansenii* ATCC 23769. For the experimental groups, 1.0% (w/v) glucose was replaced by 1.0% (w/v) purified EPS. The chemical media with and without glucose were used as positive and negative controls, respectively. All experiments were conducted in 24 well plates with 1 mL media in each well, and the growth conditions were static at 30°C for 2 days.

3.3.6 Tensile testing

The tensile tests of cellulose pellicles were performed by a dynamic mechanic analyzer (DMA) (Q800, TA Instrument, New Castle, DE). The freeze-dried BC nanocomposites were pressed into a flat piece at 500 psi by an Instron (model 3345, Norwood, MA). The pressed samples were cut into 20 × 5 mm pieces for testing. The film/fiber tension clamps were used, and the experiments were run at 1.0% strain/min under ambient temperature (25°C). Strain (ε) was calculated by $\Delta L/L_0$ where $L_0$ is the
initial length of a sample between clamps, and \( \Delta L \) is the increased length from \( L_0 \). Stress (\( \sigma \)) was calculated by \( F/A \), where \( F \) is the force applied in Newtons and \( A \) is the cross area measured by width multiplying thickness of a sample. Young’s modulus was calculated through the linear region of Stress/Strain.

### 3.3.7 X-ray diffraction (XRD)

The crystallinity of BC nanocomposites was determined by collecting x-ray diffraction diagrams using PANalyticalX’Pert Pro multi-purpose diffractometer (Almelo, the Netherlands) with Cu K\( \alpha \) radiation generated at 45 kV and 40 mA. The lyophilized BC nanocomposites were pressed into flat pieces and mounted onto a quartz sampler holder. The data were generated in reflection mode and collected in the \( 2\theta \) range of 5 – 45° with a step size of 0.026°.

MDI Jade Software (Materials Data, Livermore, CA) was used to analyze the diffraction patterns. The crystallinity of samples was estimated by peak deconvolution method where a pseudo-Voigt function was used to fit the peak shape and area, and a broad peak at around 21.5° was assigned as the amorphous contribution (Thygesen, Oddershede, Lilholt, Thomsen, & Ståhl, 2005). The ratio of the area of all crystalline peaks and the total area of all peaks is the crystallinity.

Crystal size was calculated using the Scherrer equation (Nieduszynski & Preston, 1970) with the fitting results:

\[
B_{hkl} = \frac{KL}{\sqrt{(\Delta 2\theta)^2 - (\Delta 2\theta_{\text{inst}})^2 \cos \theta}}
\]
where $B_{hkl}$ is the average crystalline width of a specific plane ($hkl$ Miller indices); $K$ is the shape factor, and the value of 0.9 was used; $\lambda$ is the wavelength of incident X-rays ($\lambda = 0.15418$ nm); $\theta$ is the center degree of the peak; $\Delta 2\theta$ is the full width at half maximum (FWHM) of the reflection peak and $\Delta 2\theta_{\text{inst}}$ is the instrumental broadening which is 0.0018 radians for this equipment.

### 3.3.8 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of purified EPS and BC nanocomposites were collected by a Bruker FTIR system (Model Vertex V70, Bruker Billerica, MA) equipped with a diamond attenuated total reflection (ATR) accessory. Samples were mounted by a high-pressure clamp, and the spectra were the averages of 32 scans in the range of 400 – 4000 cm$^{-1}$ at a resolution of 4 cm$^{-1}$.

### 3.3.9 Field emission scanning electron microscopy (FESEM)

Lyophilized cellulose samples were sputter-coated by a thin layer of iridium for taking surface images to observe the morphology and microstructures. A Nova NanoSEM 600 filed emission scanning electron microscope (FEI, Hillsboro, OR) was used with the electron beam operating at 5 kV under high vacuum. The ribbon widths of samples were measured by ImageJ software (Schneider, Rasband, & Eliceiri, 2012) by counting more than 100 ribbons for each sample.
3.3.10 Statistical analysis

The significant difference of the results was evaluated through Student’s t-test ($p < 0.05$ or $p < 0.01$) in Minitab Statistical Software (Release 17.1, University Park, PA). Each data point was replicated at least three times independently, and the standard deviation was calculated.

3.4 Results

3.4.1 Monosaccharides analyses of EPS and BC nanocomposites

Almost 80 distinct capsular polysaccharides produced by *E. coli* have been described (Whitfield, 2006). As for the subspecies *E. coli* ATCC 35860, it was able to produce various kinds and amounts of polysaccharides, including colanic acid and polysialic acid, under different culture conditions, such as by changing the incubation temperature and the types of carbon and nitrogen sources (Navasa et al., 2009). After growing the strain in HS medium at 30°C and 250 rpm, the total EPS was harvested, and monosaccharides analysis was performed by GC-MS. Seven different monosaccharides were detected including glucuronic acid, rhamnose, fucose, mannose, galactose, glucose, and glucosamine. The molar percentage of each monosaccharide in the extracted EPS was listed in Table 3.1. When comparing types and ratios of these monosaccharides with the known polysaccharides produced by *E. coli* ATCC 35860, the extracted EPS was neither pure colanic acid which should be composed of glucose, galactose, fucose, and glucuronic acid at the ratio of 2:2:2:1 (Patel et al., 2012), nor polysialic acid which was composed of sialic acid, a nine-carbon sugar (Ferrero & Aparicio, 2010).

*G. hansenii* ATCC 23769 did not produce any significant amount of water soluble-EPS in HS medium at 30°C under static condition (Fang & Catchmark, 2015)
which was confirmed by the control BC group (Table 3.1). This characteristic makes the strain an ideal model to study the effects of extrinsic EPS on the properties of bacterial cellulose. The BC nanocomposites were produced by adding different amounts of purified EPS from *E. coli* ATCC 35860 to HS media at the beginning of cultivation. Monosaccharides analyses of BC nanocomposites were conducted by GC-MS and shown in Table 3.1. The results indicated that glucose was the only sugar in 4 mg/L EPS BC, while 25.8% of fructose and 74.2% of glucose were present in 1000 mg/L EPS BC. Overall, besides glucose, the other six monosaccharides existed in purified EPS were not detected while another sugar, fructose, was measured in 1000 mg/L EPS BC.

Table 3.1 The molar percentage of each monosaccharide in the EPS extracted from *E. coli* ATCC 35860, control BC, 4 mg/L EPS BC, and 1000 mg/L EPS BC (n = 3).

<table>
<thead>
<tr>
<th>Monosaccharides (%)</th>
<th>EPS Control BC</th>
<th>4 mg/L EPS BC</th>
<th>1000 mg/L EPS BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucuronic acid</td>
<td>1.3 ± 0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>15.2 ± 0.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fucose</td>
<td>3.6 ± 0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fructose</td>
<td>-</td>
<td>-</td>
<td>25.8 ± 0.9</td>
</tr>
<tr>
<td>Mannose</td>
<td>33.2 ± 1.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galactose</td>
<td>14.3 ± 0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>26.5 ± 2.0</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>6.0 ± 0.3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: -, not detected
3.4.2 Growth of *G. hansenii* ATCC 23769 in chemical media with EPS as the only carbon source

Since only fructose and glucose were detected in 1000 mg/L EPS BC and the other six sugars in EPS were not detectable, the hypothesis for the change is that EPS from *E. coli* ATCC 35860 could be metabolized by *G. hansenii* ATCC 23769. The growth status of *G. hansenii* ATCC 35860 in chemical media with purified EPS as the only carbon source was tested (Figure 3.1). As shown in Figure 3.1a and b, there was no pellicle formed in the negative control while bacterial cellulose was produced in the positive control, which indicated that the chemical medium could be used to test whether a carbon source could support the growth of *G. hansenii* ATCC 35860. The cellulose pellicle formed (Figure 3.1c) revealed that *G. hansenii* ATCC 35860 could utilize the EPS from *E. coli* ATCC 35860 for its growth.

![Figure 3.1](image_url)

Figure 3.1 Chemical media with different carbon sources for the growth of *G. hansenii* ATCC 23769. a, chemical medium without glucose, negative control; b, chemical medium (1.0% glucose), positive control; c, chemical medium replaced glucose with EPS from *E. coli* ATCC 35860 (1.0% EPS).

3.4.3 Production of cellulose after adding EPS

Researchers have found that many polymeric additives, such as xanthan gum, carboxymethyl cellulose (CMC), agar, and acetan, would inhibit the formation of large
clumps of BC and improve the production of BC by containing these polymers in culture preparation (Cheng et al., 2009; Ishida, Mitarai, Sugano, & Shoda, 2003; Zhou, Sun, Hu, Li, & Yang, 2007). After the addition of different amounts of purified EPS into HS media at the beginning of cultivation of *G. hansenii*, the yield of cellulose was measured and shown in Figure 3.2. The results revealed that there was no significant difference in cellulose production between the EPS added group and the control group. The average dry weights of cellulose pellicles were all between 0.9 and 1.2 g/L.

![Figure 3.2](image_url)

Figure 3.2 The production of cellulose in dry weight by *G. hansenii* ATCC 23769 at the end of the 5-day incubation in culture media containing 4 mg/L EPS, 8 mg/L EPS, 40 mg/L EPS, 200 mg/L EPS, 1000 mg/L EPS and control (n = 3).
3.4.4 Mechanical properties

High mechanical strength is one of the major features of BC enabling its use as an additive in paper manufacturing, as a biomedical material in wound care, and a natural nanomaterial for water filtration (Huang et al., 2014; Jozala et al., 2016). The effects of adding different amounts of EPS from E. coli ATCC 35860 on mechanical properties of cellulose pellicles were studied through tensile testing. Figure 3.3 shows the changes of mean values and standard deviations of Young’s modulus, strain at break, and stress at break at different concentrations of EPS. By adding 4 mg/L and 8 mg/L EPS into the culture media, Young’s modulus was significantly increased compared to that of the control BC, especially for the 4 mg/L EPS BC where Young’s modulus increased from 1151 MPa (control BC) to 2231 MPa. The stress at break for 4 mg/L EPS BC also significantly increased \((p < 0.01)\) from 30.0 MPa (control BC) to 51.5 MPa. With the raising of EPS concentration to 40 mg/L and 200 mg/L, no significant change was observed in Young’s modulus, strain at break, and stress at break, when comparing the values to those of the control BC. When 1000 mg/L EPS was added, Young’s modulus of the resulting pellicles was significantly decreased \((p < 0.05)\) to 687 MPa while it was 1151 MPa for the control BC. The strain at break for 1000 mg/L EPS BC significantly increased \((p < 0.05)\) from 4.5% (control BC) to 6.4%. Moreover, no significant difference was shown in stress at the break between the 1000 mg/L EPS BC and the control BC. Figure 3.3d shows the sample curves of all experimental groups during tensile testing, where a linear strain-stress response (elastic deformation) was observed at the beginning of the tests, followed with uniform plastic deformation, and ended at the rupture point.
Figure 3.3 Results of tensile tests for cellulose pellicles produced by *G. hansenii* ATCC 23769 in culture media containing 4 mg/L EPS, 8 mg/L EPS, 40 mg/L EPS, 200 mg/L EPS, 1000 mg/L EPS and a control. a, Young’s modulus; b, strain (ε) at break; c, stress (σ) at break; d, sample curves (5 ≤ n ≤ 10). *, p < 0.05; **, p < 0.01.

### 3.4.5 X-ray diffraction

The XRD data shown in Figure 3.4 reveals the crystal dimensions of cellulose pellicles produced in culture media upon adding different amounts of purified EPS. Three main characteristic peaks of cellulose for crystal plane (100), (010), and (110) (Fang & Catchmark, 2015) were all observed in each sample. Based on those data, the crystallinity and crystal size were calculated (Table 3.2). The crystallinity of cellulose pellicles was significantly decreased (p < 0.01) from 81.2% to 69.9% when 1000 mg/L EPS was
added, whereas no significant difference \((p < 0.05)\) in crystallinity was observed between the other four EPS BC and the control BC. Table 3.2 also shows that there was no significant difference \((p < 0.05)\) in \(d\)-spacing and crystal size of the three main crystal planes.

Figure 3.4 X-ray diffraction patterns of cellulose pellicles produced by \textit{G. hansenii} ATCC 23769 in culture media containing 4 mg/L EPS, 8 mg/L EPS, 40 mg/L EPS, 200 mg/L EPS, 1000 mg/L EPS and a control (\(n = 3\)).
Table 3.2 Crystallinity and crystal size of cellulose pellicles produced in culture media containing 4 mg/L EPS, 8 mg/L EPS, 40 mg/L EPS, 200 mg/L EPS, 1000 mg/L EPS and a control (n = 3).

<table>
<thead>
<tr>
<th>d-spacing (Å)</th>
<th>Control BC</th>
<th>4 mg/L EPS BC</th>
<th>8 mg/L EPS BC</th>
<th>40 mg/L EPS BC</th>
<th>200 mg/L EPS BC</th>
<th>1000 mg/L EPS BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>6.09 ± 0.04</td>
<td>6.05 ± 0.08</td>
<td>6.07 ± 0.07</td>
<td>6.06 ± 0.07</td>
<td>6.08 ± 0.06</td>
<td>6.02 ± 0.02</td>
</tr>
<tr>
<td>010</td>
<td>5.24 ± 0.04</td>
<td>5.22 ± 0.05</td>
<td>5.22 ± 0.05</td>
<td>5.23 ± 0.04</td>
<td>5.20 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>3.90 ± 0.02</td>
<td>3.89 ± 0.03</td>
<td>3.89 ± 0.03</td>
<td>3.90 ± 0.03</td>
<td>3.88 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Crystal Size (Å)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>48.00 ± 0.00</td>
<td>48.00 ± 0.82</td>
<td>49.00 ± 1.00</td>
<td>49.67 ± 0.58</td>
<td>51.00 ± 1.22</td>
<td>51.67 ± 1.53</td>
</tr>
<tr>
<td>010</td>
<td>67.00 ± 4.24</td>
<td>66.00 ± 0.82</td>
<td>71.33 ± 0.58</td>
<td>69.67 ± 2.31</td>
<td>71.00 ± 2.00</td>
<td>68.00 ± 4.36</td>
</tr>
<tr>
<td>110</td>
<td>60.00 ± 0.00</td>
<td>59.50 ± 1.00</td>
<td>61.67 ± 0.58</td>
<td>61.67 ± 0.58</td>
<td>61.67 ± 0.58</td>
<td>60.67 ± 0.58</td>
</tr>
</tbody>
</table>

**, p < 0.01

3.4.6 Fourier transform infrared spectroscopy

Figure 3.5 shows the FTIR-ATR spectra of EPS extracted from E. coli ATCC 35860 and BC nanocomposites produced by applying different amounts of EPS at the beginning of cultivation. In the case of pure EPS, the peak observed at 1645 cm\(^{-1}\) was due to the carbonyl stretching vibration (C=O), and the C–N–H bending vibration was seen at 1544 cm\(^{-1}\) (Chi & Catchmark, 2017; Pompeo & Resasco, 2002). The peaks are consistent with previous monosaccharide analyses where glucuronic acid and glucosamine were detected in the purified EPS (Table 3.1). For BC nanocomposites produced by adding purified EPS into culture media, the two peaks at 1645 cm\(^{-1}\) and 1544 cm\(^{-1}\) were both observed in all groups. Comparing the spectra of pure EPS and BC nanocomposites, the relatively smaller peak at 1645 cm\(^{-1}\) for control BC should be associated with the H–O–H angle vibration of minor water residues (Olsson & Salmen, 2004), although all samples
had been lyophilized and were kept in a desiccator before testing. The broad bands between 3650 and 3000 cm\(^{-1}\) corresponded to O–H stretching vibrations (Gu, Catchmark, Kaiser, & Archibald, 2013). The dominant peak at 3348 cm\(^{-1}\) was related to the intramolecular hydrogen bond for 3O···H–O5 (Oh et al., 2005). When 1000 mg/L EPS was added to the culture medium, the peak at 3348 cm\(^{-1}\) broadened indicating the hydrogen bonds in cellulose pellicles were disturbed (Feng, Zhang, Shen, Yoshino, & Feng, 2012).

Figure 3.5 FTIR-ATR patterns of purified EPS and cellulose pellicles produced in culture media containing 4 mg/L EPS, 8 mg/L EPS, 40 mg/L EPS, 200 mg/L EPS, 1000 mg/L EPS and a control (n = 3).
3.4.7 Morphology of BC nanocomposites

The surface morphology of BC nanocomposites was studied by SEM, and the results are shown in Figure 3.6. The ribbons from the control BC were loosely and randomly arranged with abundant space among them, forming pores with different sizes (Figure 3.6a). The surface morphology of 4 mg/L EPS BC and 8 mg/L EPS BC were both heterogeneous, forming large ribbons in some places while at other spots ribbons were similar to that of the control BC (Figure 3.6b and c). The pore sizes in the fibrillar network in the two BC nanocomposites were smaller, compared to the ones from the control BC. With increasing EPS concentrations to 40 mg/L, 200 mg/L, and 1000 mg/L, the surface morphology of BC nanocomposites became more and more homogeneous, and both large ribbons and individual fibrils could be observed (Figure 3.6d–f). Moreover, no apparent accumulation of EPS was seen in the pores nor attaching to the fibrillar network (Figure 3.6b–f).

Figure 3.6 Surface FESEM images of cellulose pellicles produced in culture media containing EPS. a, control; b, 4 mg/L EPS BC; c, 8 mg/L EPS BC; d, 40 mg/L EPS BC; e, 200 mg/L EPS BC; f, 1000 mg/L EPS BC.
To better understand the effects of purified EPS on ribbon widths, a histogram distribution was generated by counting more than 100 ribbon widths from three independent samples for each group in Figure 3.6, and the results are shown in Figure 3.7. The control BC (Figure 3.7a) showed an average ribbon width of 54 nm and a mode of ribbon width distributed in the range of 40 and 60 nm, which agreed with the typical morphology of BC (Fang & Catchmark, 2014). The upper limit of the control BC ribbon width lay between 120 and 140 nm. As shown in Figure 3.7b, no significant changes in the average or upper limit of ribbon width were observed for the 4 mg/L EPS BC to that of the control BC. However, the mode of ribbon width for the 4 mg/L EPS BC decreased to the range of 20–40 nm. With the EPS concentration increased to 8 mg/L, 40 mg/L, 200 mg/L, and 1000 mg/L, the modes of ribbon width for those BC nanocomposites were still in the range of 20–40 nm, whereas the upper limits of ribbon width were extended to 200–280 nm.
Figure 3.7 Histogram of ribbon widths on the surface of cellulose pellicles produced in culture media containing EPS. a, control; b, 4 mg/L EPS BC; c, 8 mg/L EPS BC; d, 40 mg/L EPS BC; e, 200 mg/L EPS BC; f, 1000 mg/L EPS BC.
3.5 Discussion

3.5.1 Effects of EPS on BC production

In order to improve the properties and expand the application of bacterial cellulose, applying additional materials including additives or reinforcement materials to the culture media has been extensively studied (Cheng et al., 2009; Erbas Kiziltas, Kiziltas, Blumentritt, & Gardner, 2015; Jung et al., 2010; S.-P. Lin et al., 2016; Wu & Liu, 2012). The improvement of BC production and characteristic modifications of BC in crystallinities, porosities, and surface area, have been achieved by adding extrinsic polysaccharides to the culture media, such as carboxymethyl cellulose (CMC), chitosan, agar, and xanthan gum (Dayal & Catchmark, 2016; Stumpf et al., 2018). Unlike the EPS mentioned above, which would not be metabolized by BC-producing strains and maintained their existent in the fibrillar network, the EPS extracted from E. coli ATCC 35860 can be used as a carbon source by G. hansenii ATCC 23769 (Figure 3.1). The monosaccharide analysis of 1000 mg/L EPS BC shows glucose and fructose at a molar ratio of 74.2% and 25.8%, respectively, while the other six sugars from the added EPS were not detected (Table 3.1). This result implies that fructose is a metabolite of G. hansenii ATCC 23769 upon its utilization of EPS from E. coli ATCC 35860. Since fructose is highly water soluble and the BC nanocomposites have been extensively purified with the hot alkali solution and distilled water, it is unlikely that the fructose with a high molar ratio at 25.8% in 1000 mg/L EPS BC is in monosaccharide form. Other possible forms for the appearance of fructose could be a disaccharide, such as sucrose, or homopolymers of fructose including levan, whose main linkage is β(2→6), and inulin, where the primary linkage is β(2→1) (Tajima et al., 1997). According to Van Laere and
Van den Ende (2002), inulin was mainly produced by dicot species, whereas Tajima et al. (1997) have discovered that *Acetobacter xylinum* NCI 1005 could produce a water-soluble polysaccharide, levan, when growing on sucrose. Therefore, to explain the presence of fructose, levan is the most likely product produced by *G. hansenii* ATCC 23769.

The monosaccharide analysis of 4 mg/L EPS BC suggested it consisted of 100% glucose while fructose and the other six sugars from the added EPS were not detected (Table 3.1). The results may be caused by low signal levels from other sugars, making them unidentifiable in the noise (Stein, 1999), since glucose is the dominant sugar (at least 99.6% (w/w), assuming 1.0 g/L cellulose was produced in this case) in the hydrolysate of 4 mg/L EPS BC. As the FTIR-ATR spectra shown in Figure 3.5 for 4 mg/L EPS BC, the two peaks at 1645 cm\(^{-1}\) and 1544 cm\(^{-1}\) revealed the presence of added EPS but could not exclude the presence of fructose since its carbonyl group also showed a peak around 1645 cm\(^{-1}\) (Ahmed, Kalla, Uppuluri, & Anbazhagan, 2014). Besides, the two peaks at 1645 cm\(^{-1}\) and 1544 cm\(^{-1}\) were both observed for all BC nanocomposites indicating the presence of added EPS in those samples (Figure 3.5). However, the monosaccharides comprising the EPS were not detectable under two extreme conditions, 4 mg/L EPS BC and 1000 mg/L EPS BC, suggesting a low concentration of pure EPS in those BC nanocomposites.

Overall, with the presence of purified EPS from *E. coli* ATCC 35860, a minor portion of the EPS can be integrated into the cellulose fiber network, while the remaining EPS will be metabolized by *G. hansenii* ATCC 23769 for the production of another
polysaccharide, most probably levan. The addition of purified EPS will not affect the yield of cellulose by *G. hansenii* ATCC 23769 (Figure 3.2).

### 3.5.2 Effects of EPS on the characteristics of BC nanocomposites

The additional materials added in the culture media could modify the properties of BC pellicles through different proposed mechanisms, including inhibition of crystallization process (Benziman, Haigler, Brown, White, & Cooper, 1980), increasing the viscosity of culture media (Bae, Sugano, & Shoda, 2004), and disrupting the alignment of physically aggregated cellulose crystals (Fang & Catchmark, 2014).

Cellulose synthesis in cells is a multi-step reaction involving particular enzymes, catalytic complexes and regulatory proteins, which make the structure of cellulose easily influenced by its biosynthesis processes (Reiniati et al., 2017). After glucose is polymerized into a β-1,4 glucan chain and extruded out of the cell membranes, the assembly of glucan chains into higher-order microfibrils and ribbons would occur through the combination of different processes including co-crystallization, physical aggregation, and bundling (Fang & Catchmark, 2014). Specifically, around 10-16 glucan chains form the 1.5 nm sub-elementary fibrils which are too small to crystallize into cellulose (Zaar, 1977). It is proposed that three adjacent sub-elementary microfibrils compose the 3.5 nm elementary microfibrils, formed through co-crystallization to produce 6-7 nm microfibrils and physical aggregation to form cellulose ribbons with a typical width between 40-60 nm. Among these processes, crystallization and polymerization are considered as cell-directed, coupled processes, and the rate of polymerization is determined by the crystallization rate (Haigler, White, Brown, &
Cooper, 1982). Other processes are recognized as self-directed through hydrogen bonding or glucan stacking interactions.

The XRD results (Table 3.2) suggest that no significant differences in d-spacing and crystal size were observed between the BC nanocomposites and the control BC. Therefore, the purified EPS from *E. coli* ATCC 35860 and the other EPS produced by *G. hansenii* ATCC 23769 will not significantly affect crystallization or co-crystallization processes during cellulose synthesis. However, the crystallinity of 1000 mg/L EPS BC had significantly decreased (*p* < 0.01) from 81.2% to 69.9% (Table 3) and the average ribbon width had a significant increase (*p* < 0.01) of 14 nm compared to that of the control BC (Figure 3.7). This phenomenon had also been reported by Fang and Catchmark (2014) when adding 1 g/L hard to extract EPS (HE-EPS) into culture media. Based on these findings, a hypothesis for cellulose ribbon synthesis under the influence of highly concentrated EPS is proposed and shown in Figure 3.8c. For pure BC, physical aggregation of crystal microfibrils through London dispersion forces between their stacked heterocyclic rings (Ross, Mayer, & Benziman, 1991) will generate a ribbon shown in Figure 3.8a. It is hypothesized that when *G. hansenii* is provided with 1000 mg/L EPS in the growing media, it produces additional different EPS, most likely levan (or similar EPS). Levan is a β(2→6) linked homopolymer of fructose known to be produced by many microorganisms including *Acetobacter*, *Lactobacillus*, *Pseudomonas*, and *Streptococcus* (González-Garciniño, Tabenero, Domínguez, Galán, & Martin del Valle, 2017). Levan is likely to have a strong affinity to cellulose as it is a β-linked linear polymer. Studies on the impact of such additives like glucomannan have shown they are able to adhere to nascent microfibrils (Iwata, Indrarti, & Azuma, 1998; Tokoh, Takabe,
Fujita, & Saiki, 1998). Levan directly interrupts the cellulose-cellulose stacking interactions between the BC microfibril crystal planes. The presence of this EPS on the surface of crystal microfibrils results in bundling, causing the average and range of ribbon width to increase (Figure 3.7f) and results in less strictly aligned microfibrils. Besides the influence on the microfibril bundling process, the presence of the EPS also disturbs the intramolecular hydrogen bonding, as indicated by a broad peak shown at 3348 cm\(^{-1}\) in Figure 3.5. These combined effects explain the significant decline in overall crystallinity (Table 3.2). If significant EPS is present on the otherwise connecting surfaces of the BC crystal microfibril (plane A shown in Figure 3.8), the increase in ribbon width would be interrupted at that crystal plane resulting in thinner ribbons than the control BC (Figure 3.7f) and a decrease in Young’s modulus (Figure 3.3a). A decrease in Young’s modulus is typically accompanied with an increase in strain at break, as observed in Figure 3.3b for 1000 mg/L EPS BC. However, the improvement in strain at break, in this case, may be associated with specific changes in the BC-EPS structure. Specifically, the EPS-microfibril interaction may be much weaker than microfibril-microfibril interactions. The EPS may be able to bundle microfibrils, but the geometry of the EPS at the interface and degree of interaction is likely quite different. For example, the density of hydrogen bonding or the degree to which stacking interactions occur may be much less. Thus, when a load is applied to the composite, the EPS may allow microfibrils to slip relative to each other in response resulting in increased strain before the break. This slip type behavior has been hypothesized previously to explain, for example, how plant cell walls expand under the influence of expansions on xyloglucan-cellulose complexes (Cosgrove, 2000). The EPS could also accumulate at the amorphous
regions of microfibrils and provide additional connections among the glucan chains which may explain the significant increase in strain at break for 1000 mg/L EPS BC (Figure 3.3b).

When reducing the EPS concentrations to a lower end, such as 4 mg/L and 8 mg/L, which was seldom reported by other researchers, no significant changes in crystallinity, d-spacing, or crystal size were observed (Table 3.2). However, the mode of ribbon width was decreased to 20–40 nm (Figure 3.7b and c) and Young’s modulus had significantly increased compared to that of the control group (Figure 3.3a). The hypothesis for this phenomenon is shown in Figure 3.8b. Since the purified EPS from *E. coli* ATCC 35860 also possess abundant hydroxyl groups, its presence in media can provide additional hydrogen bonding replacing the dispersion forces. Due to the low concentration of EPS, it will be widely distributed, and only a few crystal planes will be attached with the EPS, which result in the additional formed hydrogen bonds not only compensating for the strength loss from disturbing dispersion forces but also provide additional strength. Otherwise, the EPS could be in coil forms providing spaces for their accumulation in one crystal plane, as the plane A shown in Figure 3.8. Further aggregation of crystal microfibrils will be stopped at plane A, which would result in thinner ribbons. This suggests a new method to increase the tensile strength of BC while keeping its crystallinity and crystal size the same as that of the pure BC.
Figure 3.8 Schematic representation of cellulose ribbon synthesis by *G. hansenii* ATCC 23769 in culture media containing different amounts of EPS from *E. coli* ATCC 35860. a, cross-section of a ribbon in control medium; b, cross-section of a ribbon in media containing low concentrations of EPS (4 mg/L and 8 mg/L); c, cross-section of a ribbon in media containing high concentrations of EPS (1000 mg/L).

### 3.6 Conclusion

In this research, the effects of EPS from *E. coli* ATCC 35860 on the mechanical properties of BC nanocomposites were systematically studied. When *E. coli* ATCC 35860 was grown in HS media at 250 rpm under 30 °C, the EPS produced were composed of seven sugars, including glucuronic acid, rhamnose, fucose, mannose, galactose, glucose, and glucosamine. Under the influence of purified EPS in HS media, *G. hansenii* ATCC 35860 could metabolize it and produce another kind of EPS composed of fructose. In addition, a minor part of the added EPS from *E. coli* ATCC 35860 was incorporated into the cellulose fibrillar network.
Since both the EPS from *E. coli* ATCC 35860 and another EPS produced by *G. hansenii* ATCC 23769 possess abundant hydroxyl groups to interact with the BC glucan chains, their effects could be considered as one mixture of EPS. The addition of 1000 mg/L purified EPS into the culture media resulted in the interruption of cellulose-cellulose stacking interactions during the physical aggregation of crystal microfibrils, reducing the overall crystallinity without impacting the crystallization or co-crystallization process during cellulose synthesis. When decreasing the concentration of purified EPS to 4 mg/L or 8 mg/L, a low end which has been seldom reported, Young’s modulus of the two samples was significantly enhanced compared to that of the control BC, while the crystal size was not affected. This provides a new method for maintaining the crystallinity and crystal size of BC while improving the tensile strength.

3.7 Acknowledgments

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3.8 References

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Chapter 4

Enhanced mechanical properties of bacterial cellulose produced by co-culturing *Gluconacetobacter hansenii* and *Escherichia coli* under static conditions

4.1 Abstract

Including additives in the culture media during bacterial cellulose (BC) biosynthesis is a traditional method to produce BC-based nanocomposites. This study examines a novel fermentation process, which is to co-culture *Gluconacetobacter hansenii* with *Escherichia coli* under static conditions, to produce BC pellicles with enhanced mechanical properties. The mannose-rich exopolysaccharides (EPS) synthesized by *E. coli* were incorporated into the BC network and affected the aggregation of co-crystallized microfibrils without significantly changing the crystal sizes of BC. When co-culturing *G. hansenii* ATCC 23769 with *E. coli* ATCC 700728, which produced a low concentration of EPS at 3.3 ± 0.7 mg/L, but the BC pellicles exhibited a Young’s modulus of 4,874 ± 1144 MPa and a stress at break of 80.7 ± 21.1 MPa, which are 81.9% and 79.3% higher than those of pure BC, respectively. The growth dynamics of the two co-cultured strains suggested that the production of EPS and BC were enhanced through co-culturing fermentation.
4.2 Introduction

Bacterial cellulose (BC) is a sustainable and renewable material which is composed of D-glucose units connected through β-1,4 glycosidic bonds (Hai & Sugimoto, 2018; Ribeiro-Viana, Faria-Tischer, & Tischer, 2016). Its high crystallinity, strong mechanical properties, highly hydrophilic surface, excellent biocompatibility, and non-toxic properties have motivated intense investigation of its application to papermaking, wastewater treatment, food packaging, cosmetics, and especially in medical fields including use as artificial skin, vascular grafts, cardiovascular implants and a material for bone tissue regeneration, wound dressing and drug delivery (Araujo et al., 2018; W. Hu, Chen, Yang, Li, & Wang, 2014; Klemm et al., 2006; Lee et al., 2015; Muller et al., 2013; Reiniati, Hrymak, & Margaritis, 2017; Shi, Zhang, Phillips, & Yang, 2014; Stumpf, Yang, Zhang, & Cao, 2018).

Due to the unique micro/nano-porous three-dimensional network of BC and the abundant hydroxyl groups on its surface, various modifications have been explored in order to expand the scope of BC applications (Gandini & Belgacem, 2015; Jozala et al., 2016). One of the traditional modification methods is to include additives in the culture media during the biosynthesis of BC. The additives, including xanthan gum, chitin, pectin, agar, xyloglucan, sodium alginate, carboxymethyl cellulose (CMC), 1-methylcyclopropene (1-MCP), poly(vinyl alcohol) (PVA), polycaprolactone (PCL), and poly-3-hydroxybutyrate (PHB), have been added in the culture media to study their effects on BC properties (Cheng, Catchmark, & Demirci, 2009; Dayal & Catchmark, 2016; Figueiredo, Silvestre, Neto, & Freire, 2015; Gea, Bilotti, Reynolds, Soykeabkeaw, & Peijs, 2010; Y. Hu & Catchmark, 2010; Ruka, Simon, & Dean, 2013; Szymanska-
Chargot et al., 2017). These additives may interact with the bacterial cells or directly bind to the cellulose fibrils during its synthesis, thereby affecting the yield, structure, morphology and physical properties of BC, or endowing new properties to BC, such as antibacterial, optical, catalytic, and biomedical activities (Erbas Kiziltas, Kiziltas, Blumentritt, & Gardner, 2015; W. Hu et al., 2014). For example, with the presence of the bioplastic PHB in the culture media, BC/PHB nanocomposites were able to be synthesized due to the incorporation of PHB into the BC fibrils, resulting in better mechanical properties than neat PHB (Ruka et al., 2013). However, when applying the above method to large-scale production, the cost for producing BC-based nanocomposites would significantly increase, since purified additives are needed.

Co-culture fermentation has been widely used in fermentation processes including the production of bulk chemicals, enzymes, food and food additives, antimicrobial substances, and microbial fuel cells (Goers, Freemont, & Polizzi, 2014; Sharma, Xu, & Qin, 2017). For example, the production of polydextrans uses *Rhodotorula rubra*, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Simova, Frengova, & Beshkova, 2004). During co-culture, degradation and metabolization of substrates occur based on the combined metabolic activities of the strains which may result in increased yield, improved control of product qualities and the possibility of utilizing cheaper substrates (Bader, Mast-Gerlach, Popovic, Bajpai, & Stahl, 2010). If co-culture was applied to the production of BC-based nanocomposites, the additives, such as xanthan gum, alginate, gellan, and pullulan, could be synthesized by the co-cultured microorganisms (Freitas, Torres, & Reis, 2017) and thus reduce the production cost. However, little has been studied about co-culturing BC producing strains with other...
microorganisms. Seto et al. (2006) had co-cultured *Gluconacetobacter xylinus* and *Lactobacillus mali* in corn steep media and observed a threefold higher cellulose yield comparing to the one from the monoculture of *G. xylinus*. The authors concluded that cell-cell interactions assisted by the exopolysaccharides (EPS)-producing *L. mali* promoted the cellulose production in *G. xylinus*.

In the present study, *Gluconacetobacter hansenii* ATCC 23769 was used for BC synthesis since it could not synthesize any significant amount of water-soluble EPS, which made it an ideal model to study the interactions between cellulose and other materials (Fang & Catchmark, 2015). Inspired by the EPS extracted from *Escherichia coli* (E. coli) ATCC 35860 that improved the tensile strength of BC (Liu & Catchmark, 2018), *E. coli* ATCC 35860 and *E. coli* ATCC 700728 were chosen for EPS production during co-culture. The compositions of these EPS were analyzed by gas chromatography-mass spectrometry. The BC pellicles were synthesized through co-culturing *G. hansenii* and *E. coli* under static conditions. The morphology of the cellulose pellicles was observed by field emission scanning electron microscope, the structure was characterized by Fourier transform infrared spectroscopy and x-ray diffraction, and the mechanical properties were measured by tensile tests. In order to further understand the growth status of two strains during co-culture, the glucose concentration in the media, colony forming units for each strain, biomass, the production of BC and EPS were monitored through the whole process of co-culture.
4.3 Materials and methods

4.3.1 Strains and culture media

_Gluconacetobacter hansenii_ ATCC 23769, _Escherichia coli_ ATCC 700728, and _Escherichia coli_ ATCC 35860 were obtained from the American Type Culture Collection (ATCC). The co-culture of _G. hansenii_ and _E. coli_ was performed in standard Hestrin-Schramm (HS) medium containing 20.0 g of glucose, 5.0 g of bacterial peptone, 5.0 g of yeast extract, 2.7 g of sodium phosphate dibasic, 1.15 g of citric acid, and 1.0 g of magnesium sulfate per liter of water with the pH adjusted to 5.0 by hydrochloric acid (Hestrin & Schramm, 1954). The HS agar media was prepared by adding 1.5% (w/v) agar in the standard HS media.

4.3.2 Purification of EPS produced by _E. coli_

EPS was collected after the monoculture of _E. coli_ in HS media under static conditions and 30°C for 120 hours. The method used for EPS purification was described by Obadia et al. (2007). Briefly, the cell culture was heated to 100°C and kept for 15 min to denature EPS-degrading enzymes. After cooling down, the cells in the media were removed by centrifuging at 13,200 × g at 4°C for 30 min, and the EPS in every 50 mL of supernatant was precipitated by adding three volumes of ethanol. The mixture was kept at 4°C overnight and centrifuged at 13,200 × g at 4°C for 30 min. The resulting pellet was re-dissolved in 5 mL distilled water, dialyzed with a 3.5 kDa molecular weight cutoff membrane (SnakeSkin Dialysis Tubing, Thermo Scientific) for 48 h against distilled water, and lyophilized (model Freezoom 2.5 L, Labconco, Kansas, MO). Five milliliters of 10% (v/v) trichloroacetic acid (TCA) were used to re-dissolve the freeze-dried powder.
and to precipitate residual polypeptides. The mixture was centrifuged again under the same conditions as described above. The supernatant was dialyzed against distilled water for 5 days, and the Bradford assay was applied to determine the protein concentration in the dialyzed EPS solution to ensure the results were less than 1%. Finally, the dialyzed solution was freeze-dried, and the resulting EPS powder was accurately weighed on an analytical balance (Mettler AE240, Greifensee, Switzerland) and stored at 4°C. The EPS extracted from *E. coli* ATCC 700728 and *E. coli* ATCC 35860 were named EPS7 and EPS3, respectively.

4.3.3 Monosaccharide composition analysis of EPS from *E. coli*

The monosaccharide analysis of EPS from *E. coli* was measured by gas chromatography-mass spectrometry (GC-MS). The sample preparation was performed in two steps: (a) the hydrolysis of EPS with 2 M trifluoroacetic acid (TFA) to obtain monosaccharides and (b) the trimethylsilyl (TMS) derivatization of monosaccharides. The detailed processes were described in the previous article (Liu & Catchmark, 2018). The derivatized monosaccharides were measured with an Agilent 7890A gas chromatography system coupled with an Agilent 5975 mass spectrometer (Agilent Technologies, Santa Clara, CA). A DB-5 (5%-Phenyl-methylpolysiloxane) capillary GC column (30 m × 0.25 mm I.D. × 0.25 µm film thickness, Agilent Technologies) was utilized with helium as the carrier gas at a constant flow rate of 1 mL/min. In order to gather the relative response factor (RRF) and calculate the molar percentage of each monosaccharide, the sugar standards (1 mg/mL, Sigma) were also tested under the same conditions as described above.
4.3.4 Co-culture conditions and purification of cellulose pellicles

At the beginning of co-culture, the colony forming unit (CFU) ratio between *G. hansenii* and *E. coli* was set as 1:1. In order to keep this ratio for every experimental group, the primary inoculum for each strain was prepared. For *G. hansenii*, 50 mL HS media, 170 µL thawed frozen stock, and 170 µL cellulase (Sigma) were mixed and shaken at 250 rpm and 30°C until the optical density (OD) measured at 600 nm reached 0.90. The cell culture was washed three times with an equal volume of fresh HS media to remove the cellulase. For *E. coli*, 50 mL HS media and 500 µL thawed frozen stock were shaken at 30°C and 250 rpm until the values of OD$_{600nm}$ reached 0.76 for *E. coli* ATCC 700728 or 0.81 for *E. coli* ATCC 35860 where these strains were at their early log phase.

When starting co-culture, 1 mL primary inoculum of *G. hansenii* with 0.1 mL of *E. coli* ATCC 700728 or 0.4 mL of *E. coli* ATCC 35860 were inoculated to 100 mL HS media in 250 mL Erlenmeyer glass flasks. After keeping the co-culture at 30°C and static conditions for 5 days, the cellulose pellicles were collected and treated with 0.1 M NaOH solution at 80°C for 1 hour. The alkali treatment was repeated once followed by a deionized (DI) water rinse until a pH of 7.0 was achieved. The purified pellicles were freeze-dried, accurately weighed, and stored in a desiccator for further analyses. The BC produced from co-culturing *G. hansenii* and *E. coli* ATCC 700728 was named C23769 & 700728 BC while the one from *G. hansenii* and *E. coli* ATCC 35860 was labeled as C23769 & 35860 BC. A control experiment of monoculture *G. hansenii* was performed simultaneously.
4.3.5 Characterization of cellulose pellicles from co-culture

The morphology of cellulose samples was measured by a Nova NanoSEM 600 (FEI, Hillsboro, OR) field emission scanning electron microscope (FESEM). Freeze-dried samples were sputter-coated by a thin layer of iridium, and the electron beam was operated at 5 kV under high vacuum. The distance between two bacterial cellulose layers in the cross-section images was measured by ImageJ software (Schneider, Rasband, & Eliceiri, 2012) by counting a total of 15 times from three duplicates. The Fourier transform infrared spectroscopy (FTIR) spectra were collected through a Bruker Vertex V70 (Billerica, MA) equipped with a diamond attenuated total reflection (ATR) accessory. Samples were mounted by a high-pressure clamp, and the spectra were the averages of 32 scans in the range of 400 – 4000 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\). The crystallinity of cellulose pellicles was determined by collecting X-ray diffraction (XRD) diagrams using PANalyticalX’Pert Pro multi-purpose diffractometer (Almelo, the Netherlands) with Cu K\(\alpha\) radiation generated at 45 kV and 40 mA. The freeze-dried pellicles were pressed into flat pieces and mounted onto a quartz sample holder. The data were generated in reflection mode and collected in the 2\(\theta\) range of 5 – 45° with a step size of 0.026°. MDI Jade Software (Materials Data, Livermore, CA) was used to analyze the diffraction diagrams. The crystallinity of samples was estimated by peak deconvolution method where a pseudo-Voigt function was used to fit the peak shape and area, and a broad peak at around 21.5° was assigned as the amorphous contribution (Thygesen, Oddershede, Lilholt, Thomsen, & Ståhl, 2005). The information of crystal size was calculated by the Scherrer equation (Nieduszynski & Preston, 1970):
\[ B_{hkl} = \frac{K\lambda}{\sqrt{(\Delta2\theta)^2 - (\Delta2\theta_{\text{inst}})^2 \cos\theta}} \]

where \( B_{hkl} \) is the average crystalline width of a specific plane (\( hkl \) Miller indices); \( K \) is the shape factor, and the value of 0.9 was used; \( \lambda \) is the wavelength of incident X-rays (\( \lambda = 0.15418 \text{ nm} \)); \( \theta \) is the center degree of the peak; \( \Delta2\theta \) is the full width at half maximum (FWHM) of the reflection peak and \( \Delta2\theta_{\text{inst}} \) is the instrumental broadening which is 0.0018 radians for this instrument. The tensile tests of cellulose pellicles produced through co-culturing were measured by an Instron (model 3345, Norwood, MA). The lyophilized cellulose pellicles were pressed into a flat piece at 500 psi by the Instron and cut into 60 × 10 mm pieces for testing. The film tension clamps were used and the experiments were run at 1.0 mm/min under ambient temperature (25°C). Strain (\( \varepsilon \)) was calculated by \( \Delta L/L_0 \) where \( L_0 \) is the initial length of a sample between clamps, and \( \Delta L \) is the increased length from \( L_0 \). Stress (\( \sigma \)) was calculated by \( F/A \), where \( F \) is the force applied in Newtons and \( A \) is the cross area measured by width multiplying thickness of a sample. Young’s modulus was calculated using the linear region of stress vs. strain data.

### 4.3.6 Characterization of biological parameters during co-culture

The biological parameters were measured during co-culturing \( G. \ hansenii \) ATCC 23769 and \( E. \ coli \) ATCC 700728 with monitoring the monoculture of these strains as control groups. The glucose concentration in the culture media was calculated by the glucose assay kit (Sigma). In order to investigate the viable strains during co-culture, the CFU for each strain were counted. Since the strains were trapped in the cellulose pellicles, a dispersion process conducted by Ultra-Turrax (Model T 25D, Germany) at 8 k
for 2 min was applied to break the pellicle into small fibers, followed by adding 3% cellulase. The mixture was then kept at 150 rpm and 4°C for 24 hours. A serial dilution was conducted for the following CFU counting. For E. coli, the serially diluted samples were coated on HS agar plates. The colonies appeared in 24 hours were E. coli only whereas G. hansenii would form visible colonies between 3 to 4 days. For G. hansenii, the serially diluted samples were coated on HS agar plates with 2% (w/v) acetic acid. Due to the presence of acetic acid, E. coli would not grow in 7 days, and the colonies appeared between 4 to 5 days were G. hansenii. The biomass during co-culturing was also measured. After the cellulose was fully hydrolyzed through the processes described above, the culture media were centrifuged at 12,000 × g for 5 min. The resulting pellet was washed with distilled water and dried in an oven at 80°C for 24 hours and weighed on an analytical balance. The dry weight of cellulose pellicles during co-culture was measured every 24 hours. After the pellicles were harvested, the treatment for purification followed the steps described in 2.4. The EPS production during co-culture was tested every 24 hours. In order to release the EPS from the cellulose network, the pellicles were firstly hydrolyzed with cellulase and purification was performed according to the procedures in 4.3.2.

4.3.7 The growth of G. hansenii ATCC 23769 in chemical media with EPS as the only carbon source

The chemical medium composed of 10.0 g of glucose, 3.0 g of (NH₄)₂SO₄, 1.0 g of KH₂PO₄, and 2.0 g of Na₂HPO₄·12H₂O (pH 5.0) (Son et al., 2003), was used to test whether G. hansenii ATCC 23769 could utilize the EPS extracted from E. coli to grow
and produce cellulose. For the experimental groups, glucose was replaced entirely by the same amounts of purified EPS from *E. coli* ATCC 700728. The chemical media with and without glucose were used as positive and negative controls, respectively. All experiments were performed in 24 well plates with 1 mL media in each well, and the growth conditions were static at 30°C for 2 days.

### 4.3.8 Statistical analysis

The significant difference between two results was evaluated by Student’s t-test ($p < 0.05$ or $p < 0.01$) in Minitab Statistical Software (Release 17.1, University Park, PA). Each data point was replicated at least three times, and standard derivation (SD) was calculated for the error bar shown in figures.

### 4.4 Results and discussion

#### 4.4.1 EPS produced by *E. coli* ATCC 700728 and *E. coli* ATCC 35860

The culture conditions including pH, temperature, presence of agitation, and the compositions of culture media are all able to affect the yield and compositions of EPS produced by *E. coli* (Navasa, Rodriguez-Aparicio, Martinez-Blanco, Arcos, & Ferrero, 2009; Whitfield, 2006), as well as the production and form of BC synthesized by *G. hansenii* (Campano, Balea, Blanco, & Negro, 2015; Mikkelsen, Flanagan, Dykes, & Gidley, 2009). When co-culturing *G. hansenii* and *E. coli* together, an optimized culture condition, which meets the production demands for both BC and EPS by these strains, needs to be considered. For *G. hansenii*, it can produce BC in pellicle form under static
conditions or in other forms, including fibers and pellets under different agitation conditions (Y. Hu, Catchmark, & Vogler, 2013; Islam, Ullah, Khan, Shah, & Park, 2017). Among these forms, BC pellicles usually present stronger mechanical properties and higher water holding capacity due to an entire cellulose network and more extensive interior surface area (Huang et al., 2014), which is preferred for many applications. Thus, the static condition was chosen in this study. The optimal pH and temperature for *G. hansenii* to grow and produce BC in HS media are at 5-6 and 28-30°C, respectively (Campano et al., 2015; Matsuoka, Tsuchida, Matsushita, Adachi, & Yoshinaga, 1996). Considering the narrow culture conditions required by *G. hansenii*, it is difficult to vary the production of EPS by utilizing one kind of *E. coli* strain. In this research, *E. coli* ATCC 700728 and *E. coli* ATCC 35860 were both used to co-culture with *G. hansenii*, where the former strain could produce 3.3 ± 0.7 mg/L EPS while the later one could synthesize 41.4 ± 3.8 mg/L EPS under the same static conditions (Table 4.1). The compositions of EPS7 were mannose, galactose, glucose, and glucosamine while EPS3 contained 1.7% rhamnose in addition to the above four monosaccharides (Table 4.1). However, the main component in these EPS was both mannose (over 90% in molar percentage), which allowed them to be compared in this study.

Mannose-rich EPS are known to exhibit a strong affinity to cellulose. Whitney, Brigham, Darke, Reid, and Gidley (1998) predicted strong interactions between mannose-based polysaccharides and cellulose due to the similarity of mannose to cellulose in stereochemistry with the only difference being the configuration of the hydroxyl group at the C2 position. In practice, during the pretreatment of lignocellulosic materials, dissolved mannan could even be re-adsorbed onto the cellulose (Wang, Li,
Yang, Wang, & Zhang, 2016). Research by Fang and Catchmark (2014) obtained hard to extract EPS (HE-EPS) from the BC films synthesized by *Gluconacetobacter xylinus* ATCC 53582 with 4 M NaOH aqueous solutions and the main components of the HE-EPS, with a value of 75.4%, was also mannose.

Table 4.1 The concentration of EPS and the molar percentage of each monosaccharide when monoculture *E. coli* ATCC 700728 or *E. coli* ATCC 35860 under static conditions for 120 hours (n = 3).

<table>
<thead>
<tr>
<th>Monosaccharides (%)</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>Mannose</td>
</tr>
<tr>
<td>EPS7</td>
<td>95.4 ± 0.6</td>
</tr>
<tr>
<td>EPS3</td>
<td>1.7 ± 0.0</td>
</tr>
</tbody>
</table>

Note: -, not detected

### 4.4.2 Characterization of cellulose pellicles from co-culture

The surface and cross-section morphologies of control BC and cellulose pellicles from co-culture were characterized by FESEM (Figure 4.1). The surface of the control BC produced by the monoculture of *G. hansenii* exhibited a randomly arranged and porous cellulose network (Figure 4.1a) which agrees with the typical morphology reported for BC (Chi & Catchmark, 2017). When co-culturing *G. hansenii* with *E. coli* ATCC 700728, the surface of the C23769 & 700728 BC became heterogeneous, forming a denser cellulose network and larger ribbons in some places as compared to the control BC (Figure 4.1b). With changing the co-cultured strain to *E. coli* ATCC 35860, which resulted a higher concentration of EPS, a much denser and less porous cellulose network structure was observed as compared to the C23769 & 700728 BC (Figure 4.1c). The
significant role of EPS in regulating the bundling process of BC microfibrils which would result in larger bundles has been reported previously (Fang & Catchmark, 2015; Liu & Catchmark, 2018). It was hypothesized that the EPS coated the surface of co-crystallized microfibrils promoting bundling. For the cross-section images, a clear layer-by-layer cellulose structure was observed in the control BC (Figure 4.1d). With the presence of EPS3 by co-culturing with *E. coli* ATCC 700728, the microfibrils between the layers were bound together, and a part of the porous regions between adjacent layers was filled with EPS. However, the different layers were still clearly observed (Figure 4.1e). When increasing the concentration of EPS by co-culturing with *E. coli* ATCC 35860, the boundaries between different BC layers became ambiguous, and the pore sizes were comparable to that of the control BC (Figure 4.1f).

![Figure 4.1](image)

Figure 4.1 Representative FESEM images of (a) and (d) surface and cross-section of control BC; (b) and (e) surface and cross-section of C23769 & 700728 BC; (c) and (f) surface and cross-section of C23769 & 35860 BC. The red circles indicate ambiguous boundaries between different BC layers.
To better qualify the effects of EPS on the forming of BC layers, the distance between adjacent layers was measured 15 times from three independent cellulose samples for each experimental group (Figure 4.2). For the control BC, the average distance between adjacent layers was 5.8 ± 0.8 µm, and the distribution of the distance was within the range of 5 – 10 µm which is typically reported for pure BC (Ruan et al., 2016). For the C23769 & 700728 BC, the average distance was 3.0 ± 0.5 µm which is a significant decrease ($p < 0.01$) as compared to the control BC. With increased EPS concentration, the average distance between adjacent layers for the C23769 & 35860 BC was 6.1 ± 1.0 µm, which is comparable to the values of the control BC. The possible interpretation for the different distance between layers is that a low concentration of EPS coats a part of microfibrils, which provides further bundling between EPS-cellulose and helps to reinforce the physical aggregation between co-crystallized microfibrils, resulting in a denser stacking of layers. With the presence of more EPS, the co-crystallized microfibrils will be gradually fully coated by them, leaving less space for direct cellulose-cellulose interactions, including van de Waals interactions and hydrogen bonding, which are the main forces driving aggregation of co-crystallized microfibrils. In Figure 3.1f, the boundaries between layers showed in the red circles were ambiguous while the layers could still be recognized in other regions, indicating 41.4 ± 3.8 mg/L EPS could only partially disrupt the direct cellulose-cellulose interactions. Liu and Catchmark (2018) reported the EPS from *E. coli* ATCC 35860 played a similar role in BC bundling where 4 mg/L and 8 mg/L EPS provided additional hydrogen bonding while 1000 mg/L EPS interrupted the cellulose-cellulose stacking interactions.
Figure 4.2 Box plots of distance between adjacent layers in cross section FESEM images. The box indicates the percentage between 25% and 75%. The outlier coefficient is equal to 1.5. The line crossed the box shows the average value.

The FTIR spectra of different BC samples are shown in Figure 4.3a. All the BC exhibited characteristic cellulose vibration peaks. The broad bands between 3650 and 3000 cm\(^{-1}\) corresponded to O-H stretching vibrations which was related to the hydrogen bonding in the cellulose network (Oh et al., 2005). The peak observed at 1544 cm\(^{-1}\) was contributed by the C–N–H bending vibration (Movasaghi, Rehman, & ur Rehman, 2008) which is consistent with previous monosaccharide analysis where glucosamine was detected in the EPS (Table 4.1). This peak was observed in the spectra from co-cultured BC pellicles while it was not shown from the control BC (Figure 4.3a), indicating the existence of EPS in the C23769 & 700728 BC and C23769 & 35860 BC.
XRD patterns are widely used for cellulose crystal structure analysis and crystallinity calculations. For BC samples, the three main peaks displayed at 2θ angles around 14.5, 16.7, and 22.6 corresponded to the (100), (010), and (110) crystallographic planes, respectively. The co-cultured BC and the control BC all exhibited the three main peaks (Figure 4.3b). After processing these XRD data with MDI Jade software, the values of crystallinity and crystal sizes were summarized in Table 4.2. The mean values of crystallinity for control BC, C23769 & 700728 BC, and C23769 & 35860 BC were 87.8%, 87.4%, and 84.7%, respectively, showing no significant difference. For the values of d-spacing and crystal sizes from all the BC pellicles, no significant changes were observed (Table 4.2), which confirms that the EPS did not impact the co-crystallization of microfibrils. In other words, the crystallizing processes during the synthesis of sub-elementary microfibrils and elementary microfibrils are not significantly affected by the EPS. Fang and Catchmark (2014) also reported the mannose-rich EPS, HE-EPS, interfered with the physical aggregation processes, but not the co-crystallization process.
The relatively large standard deviations in crystal sizes for the (100) and (010) planes, comparing to the values from the control BC, indicated that the EPS might have preferred planes to influence, although no significant changes were made. This preference of mannose-rich EPS was also discovered by Fang and Catchmark (2015), where the (010) plane of the cellulose microfibrils was more subject to HE-EPS modification as compared to the (100) and (110) planes.

Table 4.2 Crystallinity and crystal size of BC produced by co-culturing *G. hansenii* ATCC 23769 and *E. coli* ATCC 700728/*E. coli* ATCC 35860 (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>Control BC</th>
<th>C23769 &amp; 700728 BC</th>
<th>C23769 &amp; 35860 BC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crystallinity (%)</strong></td>
<td>87.8 ± 1.0</td>
<td>87.4 ± 2.1</td>
<td>84.7 ± 4.9</td>
</tr>
<tr>
<td><strong>d-spacing (Å)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>6.09 ± 0.01</td>
<td>6.09 ± 0.02</td>
<td>6.09 ± 0.01</td>
</tr>
<tr>
<td>010</td>
<td>5.26 ± 0.01</td>
<td>5.25 ± 0.01</td>
<td>5.25 ± 0.01</td>
</tr>
<tr>
<td>110</td>
<td>3.91 ± 0.00</td>
<td>3.90 ± 0.01</td>
<td>3.90 ± 0.00</td>
</tr>
<tr>
<td><strong>Crystal Size (Å)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>49.50 ± 0.01</td>
<td>52.33 ± 1.14</td>
<td>53.00 ± 1.73</td>
</tr>
<tr>
<td>010</td>
<td>60.00 ± 0.00</td>
<td>68.00 ± 3.61</td>
<td>68.67 ± 5.86</td>
</tr>
<tr>
<td>110</td>
<td>61.00 ± 0.00</td>
<td>61.33 ± 0.58</td>
<td>61.67 ± 1.15</td>
</tr>
</tbody>
</table>

BC has excellent mechanical properties making it useful for many applications in the medical field including wound healing treatments, as semi-permanent artificial skin, and replacing small-diameter blood vessels (Brown, Laborie, & Zhang, 2011; Huang et al., 2014; Petersen & Gatenholm, 2011). The effects of co-culturing *G. hansenii* with EPS-producing *E. coli* on the mechanical strength of cellulose pellicles were studied through tensile testing. Figure 4.4 shows the mean values and standard deviations of
Young’s modulus, stress at break, and strain at break. When co-culturing *G. hansenii* with *E. coli* ATCC 700728, which produced a relatively low concentration of EPS (3.3 ± 0.7 mg/L) during monoculture, Young’s modulus of the BC was significantly increased (*p* < 0.05) from 2,680 ± 730 MPa (control BC) to 4,874 ± 1144 MPa. The stress at break for the co-cultured BC also significantly increased (*p* < 0.05) from 45.0 ± 8.7 MPa (control BC) to 80.7 ± 21.1 MPa. The increase in these mechanical properties could be associated with the increasing density and larger ribbons (Figure 4.5) by the presence of the EPS7. With changing the co-cultured strain to *E. coli* ATCC 35860, which possessed a relatively high concentration of EPS (41.4 ± 3.8 mg/L) in monoculture, no significant change was observed in Young’s modulus, stress at break, and strain at break, when comparing these values with the ones from the control BC. We hypothesize that higher levels of EPS more fully coat the surface of co-crystallized BC microfibrils disrupting the additional aggregation and networking between cellulose and cellulose that improved the mechanical properties of the EPS-BC composite. However, the concentration of the EPS was still not high enough to exhibit negative effects on the mechanical properties. These results agree with the effects of EPS extracted from *E. coli* ATCC 35860 on the mechanical properties of BC (Liu & Catchmark, 2018). Figure 4.4d indicates the sample curves for all the three kinds of BC.
Figure 4.4 Results of tensile tests for C23769 & 700728 BC, C23769 & 35860 BC, and control one: (a) Young’s modulus; (b) stress at break; (c) strain at break; (d) sample curves; *, $p < 0.05$ (n = 5).
4.4.3 Characterization of growth dynamics during co-culture

The BC pellicles synthesized through co-culturing *G. hansenii* and *E. coli* ATCC 700728 exhibited enhanced mechanical properties while maintaining the crystallinity and crystal sizes. Traditionally, in order to produce the same modified BC pellicles, the EPS needed to be collected, purified, and finally added to the culture media to modify the cellulose. Through co-culturing, the processes for collecting and purifying the EPS were skipped which could save time and cost in production. To understand the performance of the strains during co-culture and further apply this method to produce modified BC which
could incorporate other polymers, the growth dynamics during co-culturing *G. hansenii* and *E. coli* ATCC 700728 were measured.

Figure 4.6 Characterization of biological parameters during co-culturing of *G. hansenii* ATCC 23769 and *E. coli* ATCC 700728 over 120 hours with the monoculture of the two strains as control groups: (a) glucose concentration in culture media; (b) CFU per milliliter; (c) biomass in dry weight; (d) BC pellicles in dry weigh; (n = 3). C23769 & 700728: co-culture of *G. hansenii* ATCC 23769 and *E. coli* ATCC 700728; M23769: monoculture of *G. hansenii* ATCC 23769; M700728: monoculture of *E. coli* ATCC 700728; C700728: CFU of *E. coli* ATCC 700728 during co-culturing; C23769: CFU of *G. hansenii* ATCC 23769 during co-culturing.
The changes in glucose concentration in the culture media are shown in Figure 3.6a. When *E. coli* ATCC 700728 was cultured in isolation, glucose was consumed during the first 36 hours, and then its concentration in the culture media remained constant, at around 17.2 g/L. *G. hansenii*, whether co-cultured or cultured in isolation, consumed glucose through the whole culture period. The consumption of glucose during co-culture was almost equal to the total amounts consumed through the monoculture of the two strains. The viable strains in the media during the culture period were counted by CFU (Figure 4.6b). The changes in CFU values for each strain during its monoculture or co-culture exhibited similar trends. For *E. coli* ATCC 700728, CFU values rapidly reached the maximum, at around $3.0 \times 10^8$ CFU/mL, remained constant for 48 hours, then continuously decreased to $(7.7 \pm 2.8) \times 10^3$ CFU/mL at the end of the culture period. For *G. hansenii*, CFU values gradually increased to the maximum, at around $3.5 \times 10^7$ CFU/mL, between 48 to 72 hours and then remained constant until the end of the fermentation. When comparing the time needed for each strain to reach its maximum CFU values during the monoculture and co-culture, 12 to 24 more hours were required through monoculture, which indicated that strains grew faster during co-culture.

Measured biomass includes the mass of all strains, dead and alive (Figure 4.6c). For the monoculture of *E. coli* ATCC 700728, its biomass reached the maximum value, at around 0.49 g/L, within 12 hours and remained constant until the end of the culture period. The *E. coli* could consume all the oxygen in the culture media within 12 hours (Figure 4.7), and since the culture was under static conditions, no oxygen was further supplied which could be responsible for the constant biomass after 12 hours and the declining CFU values after 48 hours (Figure 4.6b). For the monoculture of *G. hansenii*, its biomass
gradually accumulated during the whole processes of fermentation, reaching $0.56 \pm 0.06$ g/L at 120 hours. When co-culturing these two strains together, the biomass also kept increasing during the whole culture period, and its value was almost equal to the total amount produced through monoculture of each strain. The dry weight of BC pellicles produced by *G. hansenii* during its monoculture and co-culture is shown in Figure 4.6d. At 24 hours, no BC was harvested through the monoculture of *G. hansenii* while $0.02 \pm 0.01$ g/L BC was collected by co-culture. At the end of the culture period, the dry weight of BC from co-culture was 10.8% more than that of the monoculture, reaching $1.56 \pm 0.09$ g/L. Considering all the above data, the performance trends of *G. hansenii* and *E. coli* ATCC 700728 during co-culture is similar to their monoculture. However, each stain would require 12 to 24 hours less to reach their maximum CFU values, and the final production of BC is improved by 10.8% through co-culture. The possible explanations for the improvement are either due to the larger CFU values of *G. hansenii* presented in co-culture than in monoculture starting from the 48 hours (Figure 4.6b) or the gluconic acid produced by *G. hansenii*, which typically inhibits the strain from producing cellulose (Zhong et al., 2013), was partially metabolized by *E. coli*. Previously, researchers reported that *E. coli* could utilize gluconic acid as a carbon source with an even faster rate than glucose (Fan et al., 2012; Peekhaus & Conway, 1998). Since the CFU values of *G. hansenii* at 24 hours in co-culture was even less than that of the monoculture, the presence of BC at 24 hours in co-culture suggests that the promotion of cellulose production might mainly contribute to the consumption of gluconic acid by *E. coli* (Figure 4.6b).
Figure 4.7 The dissolved oxygen (DO) levels in the culture media when monoculture *E. coli* ATCC 700728 and its co-culture with *G. hansenii* ATCC 23769 (n = 3). C23769 & 700728: co-culture of *G. hansenii* ATCC 23769 and *E. coli* ATCC 700728; M700728: monoculture of *E. coli* ATCC 700728.

The concentration of EPS produced by *E. coli* ATCC 700728 during co-culture and monoculture were also measured (Figure 4.8). Under either culture condition, the maximum concentration of EPS was observed at 24 hours, which was 11.6 ± 1.0 mg/L for co-culture and 5.1 ± 0.5 mg/L for monoculture. The higher concentration of EPS during co-culture could result from the degradation and metabolization of substrates produced by the combined strains, which could benefit each other. For example, the by-product, gluconic acid, produced by *G. hansenii* is a favorable carbon source for *E. coli*. From 96 hours, the concentration of EPS measured through co-culture significantly decreased from over 10 mg/L to around 4.5 mg/L. The possible explanation for the decline of EPS concentration is its consumption by *G. hansenii*. As shown in Figure 4.9c, when the EPS extracted from *E. coli* ATCC 700728 was the only carbon source in the
chemical media, *G. hansenii* could still grow and produce cellulose. However, the production of cellulose was significantly lower than when grown with glucose (Figure 4.9b), in agreement with the less efficient production of BC in mannose as compared to glucose (Masaoka, Ohe, & Sakota, 1993).

![Figure 4.8](image1.png)

Figure 4.8 The EPS concentration by *E. coli* ATCC 700728 during its monoculture and co-culturing with *G. hansenii* ATCC 23769 (n = 3).

![Figure 4.9](image2.png)

Figure 4.9 Chemical media with different carbon sources for the growth of *G. hansenii*: (a) without glucose, negative control; (b) with 1.0% (w/v) glucose, positive control; (c) replacing glucose with 1.0% (w/v) EPS from monoculture *E. coli* ATCC 700728. The red arrow indicates the presence of cellulose.
4.5 Conclusions

Co-culturing *G. hansenii* ATCC 23769 with *E. coli* under static conditions was studied as a novel fermentation process to produce improved BC-based nanocomposites. During co-culture, the mannose-rich EPS synthesized by *E. coli* ATCC 700728 and *E. coli* ATCC 35860 were incorporated into the BC network and affected the aggregation of co-crystallized microfibrils. When co-culturing *G. hansenii* with *E. coli* ATCC 700728, which produced a low concentration of EPS at 3.3 ± 0.7 mg/L, the BC pellicles exhibited improved mechanical properties with an 81.9% increase in Young’s modulus and a 79.3% enhancement in stress at break while maintaining the crystallinity and crystal sizes as that exhibited by the control BC. The further studies in the growth dynamics of the strains revealed that the two co-cultured strains, *G. hansenii* and *E. coli* ATCC 700728, would require 12 to 24 hours less to reach their maximum CFU values during co-culture. The combined metabolic activities by the two strains played a significant role in co-culture and could be the explanation for the faster synthesis of BC pellicles in the first 24 hours and the 10.8% increase in the production of BC by *G. hansenii* through co-culture.

4.6 Acknowledgments

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4.7 References


Chapter 5

Bacterial cellulose/hyaluronic acid nanocomposites production through co-culturing *Gluconacetobacter hansenii* and *Lactococcus lactis* in a two-vessel circulating system

5.1 Abstract

Bacterial cellulose (BC) based composites have been widely studied in the biomedical field. The BC/HA (hyaluronic acid) nanocomposites in the pellicle form were directly produced through co-culturing *Gluconacetobacter hansenii* and *Lactococcus lactis* in a novel two-vessel circulating system. The concentration of HA secreted by *L. lactis* APJ3 was controlled by adjusting the constant feed rate of glucose. The dynamic growth of the two strains revealed that *L. lactis* APJ3 was mainly growing within the first 48 hours while *G. hansenii* ATCC 23769 started to grow after 48 hours. The FTIR-ATR spectroscopy proved the incorporation of HA into the cellulose network. XRD analysis indicated that the presence of HA would not affect the crystallinity of BC/HA but increase the crystalline sizes. The FESEM images showed that more ribbons within the range of 20 – 40 nm diameter and larger ribbons between the range of 180 – 360 nm diameter were observed in BC/HA composites. The strain at break and the water holding capacity of BC/HA increased with the concentration of HA. The designed two-vessel circulating system provided a new method to directly produce BC-based nanocomposites.
5.2 Introduction

Bacterial cellulose (BC) has attracted broad interests from researchers due to its high purity, biocompatibility, and excellent mechanical properties (Yang Huang et al., 2014; Rastogi, Singh, Das, Kundu, & Banerjee, 2018). The BC-based nanocomposites are extensively studied including BC/alginate (Kirdponpattara, Khamkeaw, Sanchavanakit, Pavasant, & Phisalaphong, 2015), BC/collagen/hydroxyapatite (Yan Huang et al., 2017), BC/chitosan (Johns et al., 2018), heparinized BC (Wang et al., 2018), mannosylated BC (Birkheur et al., 2017), BC/polyaniline (Shi et al., 2012), BC/pullulan (Trovatti et al., 2012), and BC/xyloglucan (Bodin et al., 2007). These BC-based nanocomposites have been applied in the biomedical field ranging from improving biocompatibility, cell attachment, and fibroblast growth to the applications as artificial blood vessels, wound dressing, and tissue regeneration.

Hyaluronic acid (HA), as a linear polysaccharide composed of disaccharide repeat units of D-glucuronic acid and N-acetyl-D-glucosamine, exhibits beneficial effects on cell growth, proliferation, and wound healing (Xiao et al., 2013). The BC/HA nanocomposites have been produced through adding purified HA in the culture medium during the biosynthesis of BC or immersing BC pellicles into the HA solutions (de Oliveira et al., 2017; Y. Li, Qing, Zhou, & Yang, 2014). As expected, the BC/HA composites manufactured through the above traditional methods improved the wound healing and tissue repair during in vivo experiments while BC acted as supporting material (Ying Li et al., 2015). However, applying purified HA in the production of BC/HA nanocomposites would significantly increase the cost due to the high price of HA in the market (L. Liu, Liu, Li, Du, & Chen, 2011).
The widely used method in biotechnology, co-culture fermentation, has provided a clue to solve the problem of high production cost. K. Liu and Catchmark (2019) manufactured BC-based nanocomposites through co-culturing *Gluconacetobacter hansenii* (*G. hansenii*) and *Escherichia coli* (*E. coli*) under static conditions. Chauhan, Badle, Ramachandran, and Jayaraman (2014) created an engineered strain, *Lactococcus lactis* APJ3 (*L. lactis* APJ3), which could secrete HA into the culture medium. By co-culturing *G. hansenii* and *L. lactis* APJ3 under proper culture conditions, the BC/HA nanocomposites could be directly formed during fermentation.

Generally, the culture conditions could be divided into agitated and static states. In agitated culture medium, the values of dissolved oxygen (DO) and pH could be monitored and adjusted when necessary. Other nutrients such as glucose could also be supplied and well mixed to support the growth of strains. Depending on the methods of agitation, the shape of synthesized BC can be in various forms, including multi-shaped pulps, fibers, and elliptical pellets (Y. Hu & Catchmark, 2010; Shi, Zhang, Phillips, & Yang, 2014). The BC produced in agitated systems presented weaker mechanical properties comparing to those synthesized in static culture (Yang Huang et al., 2014). Under static conditions, BC is produced at the air and medium interface, forming a gelatinous pellicle. These BC pellicles maintain regular shapes and keep intact three-dimensional structures. Compared with the ones synthesized under agitated conditions, BC pellicles showed higher water holding capacity due to the more extensive interior surface area (Yang Huang et al., 2014), which is more preferred in the biomedical field. However, in the static culture medium, only a few parameters could be adjusted including temperature, the initial value of pH, and initial concentrations of different nutrients. To
produce BC/HA nanocomposites in the pellicle form while the demands from two strains could be monitored and adjusted during co-culture, a novel two-vessel circulating system was developed.

In the present study, BC/HA nanocomposites in the pellicle form were directly produced in the two-vessel circulating system. The growth dynamics including values of DO, pH, and glucose concentration were measured and adjusted when necessary. By varying the constant feed rate of glucose, the amounts of HA incorporated into the BC network were controlled. The properties of the BC/HA nanocomposites were characterized by FTIR-ATR spectroscopy, XRD analysis, FESEM, tensile tests, and weight loss analysis.

5.3 Materials and methods

5.3.1 Strains and culture media

*Gluconacetobacter hansenii* ATCC 23769 was from the American Type Culture Collection (ATCC). *Lactococcus lactis* APJ3 was constructed by Chauhan et al. (2014) with *Lactococcus lactis* MG1363 and plasmid pAMJ399 from the Bioneer (Denmark). The primary culture of *G. hansenii* and *L. lactis* was prepared in standard Hestrin-Schramm (HS) medium containing 20.0 g/L glucose, 5.0 g/L bacterial peptone, 5.0 g/L yeast extract, 2.7 g/L sodium phosphate dibasic, 1.15 g/L citric acid, and 1.0 g/L magnesium sulfate with the pH adjusted to 5.0 by hydrochloric acid (Hestrin & Schramm, 1954). The HS agar medium was prepared by adding 1.5% (w/v) agar and erythromycin (Sigma-Aldrich) with a final concentration at 2 µg/mL in the standard HS medium. The co-culture of *G. hansenii* and *L. lactis* was performed in modified M17
medium containing 30 g/L glucose, 2.5 g/L casein tryptone, 2.5 g/L bacterial peptone, 5 g/L soya peptone, 2.5 g/L yeast extract, 5 g/L beef extract, 0.5 g/L ascorbic acid, 0.22 g/L magnesium sulfate, 19.1 g/L sodium-β-glycerophosphate, 3.5 g/L citric acid, and 8.97 g/L sodium phosphate dibasic. When continuously adding glucose into the fermentation system at the rate of 0.067 g/h, 0.033 g/h, and 0.017 g/h, the glucose solutions were prepared based on modified M17 medium by increasing the glucose concentration to 533 g/L, 267 g/L, and 133 g/L respectively. All the chemicals used in this study were A.C.S. grade and prepared in ultra-pure water with a resistivity of 18.2 MΩ/cm (Millipore Milli-Q UF Plus) for media and solutions.

5.3.2 Design of the two-vessel circulating system

A two-vessel circulating system was developed to allow the production of cellulose pellicles at the air-liquid interface, while supplying oxygen and glucose for the co-cultured strain, *L. lactis*, to grow and produce hyaluronic acid. The system contained two aspirator bottles (500 mL, DWK Life Sciences), where one was used for the production of cellulose pellicles (BC side) and the other one was for oxygen and glucose supplement (air side) (Figure 5.1). The two bottles were connected through their bottom outlets by a silicone tubing with an outside diameter (OD) of 1/2 inch and an inside diameter (ID) of 1/4 inch. A peristaltic pump (model L/S, MasterFlex) was used to circulate the culture media at the rate of 1.5 mL/min. The wet air supplying rate was 0.8 vvm through a circled silicone tubing with one end blocked and placing at the bottom of the culture medium. The circled silicone tubing was punched with holes at the diameter of 0.45 mm every distance of 10 mm. The inlet and outlet air was passed through 0.22
µm filters (Sigma-Aldrich). The volume of culture medium in each vessel was 110 mL. A syringe pump (PHD 2000, Harvard Apparatus) was used to continuously add glucose at the rate of 0.067 g/h, 0.033 g/h, or 0.017 g/h through pushing syringes (20 mL, DB). The size of silicone tubing for the peristaltic pump and air supplying was 1/4-inch OD × 1/8-inch ID while for syringe pump was 1/16-inch OD × 1/32-inch ID.

Figure 5.1 Schematic diagram of the two-vessel circulating system. Wet air and glucose were supplied to the air side (left vessel), mixed by the vigorous bubbling, and distributed through the circulating of culture media. Cellulose pellicle was grown at the interface of air and liquid in the BC side (right vessel). The black arrows indicate the flow direction of culture media. The dashed arrows indicate the flow direction of wet air.
5.3.3 Co-culture conditions and purification of cellulose pellicles

The colony forming unit (CFU) between *G. hansenii* and *L. lactis* was set as 1:1 at the time of inoculating for co-culturing. To maintain this ratio for each experiment group, the primary culture was prepared for each strain. For *G. hansenii*, 10 mL HS media, 34 µL thawed frozen stock, and 34 µL cellulase (Sigma-Aldrich) were mixed and shaken at 30°C and 250 rpm until the optical density (OD) at the wavelength of 600 nm reached 0.90. The cellulase in the culture medium was removed by washing the strains three times with an equal volume of fresh HS medium. The conditions of centrifuging for the collection of strains were 4,500 × g, 4°C, and 5 min. For *L. lactis*, its thawed frozen stock was firstly inoculated on HS agar plates with 2 µg/mL erythromycin to select strains containing the transferred plasmid. The plates were placed in the incubator at 30°C for 24 to 36 hours. Colonies formed on the agar plates were picked and inoculated into 5 mL fresh HS medium containing 2 µg/mL erythromycin and incubated overnight at 30°C and 250 rpm. Another 10 mL fresh HS medium containing 2 µg/mL erythromycin was inoculated with the overnight incubated medium at 1% (v/v) and shaken at 30°C and 250 rpm until the value of OD$_{600nm}$ reached 0.66 where the strains were in their early log phase. When starting co-culture, 1 mL primary culture of *G. hansenii* and 1 mL of *L. lactis* were inoculated into every 100 mL modified M17 medium which also contained 2 µg/mL erythromycin. After running the two-vessel circulating system at 30°C for 7 days, the cellulose pellicles were collected and washed with 0.1 M NaOH solution for 1 hour at 80°C. The alkali treatment was repeated once and followed by a deionized (DI) water rinse until pH 7.0 was achieved. The purified pellicles were freeze-dried (Freezoom 2.5 L, Labconco), accurately weighed (AE240, Mettler Toledo), and stored in a desiccator for
further analyses. The cellulose produced from co-culturing *G. hansenii* and *L. lactis* was labeled as Co BC while the one from monoculture *G. hansenii*, which was a control group, was labeled as 23769 BC. Monoculture of *L. lactis* APJ3 under the same conditions was performed simultaneously.

5.3.4 Analytical methods of growth dynamics

The hyaluronic acid produced through monoculture of *L. lactis* APJ3 after 7 days was purified according to the methods described by Sheng et al. (2009) and Prasad, Jayaraman, and Ramachandran (2010) with minor modification. Briefly, 1 mL of 5 % (w/v) sodium dodecyl sulphate (SDS) was added to 100 mL culture medium. The mixture was mixed and incubated at room temperature for 10 min to free the capsular hyaluronic acid and centrifuged at 4,500 × g for 5 min to remove the cells. An equal volume of 1.7 % (w/v) cetylpyridinium chloride (CPC) was added to the supernatant to precipitate HA. The CPC-HA precipitate was harvested by centrifuging at 8,000 × g for 10 min, resuspended in 2.5 mL 1 M NaCl solution, and incubated in water bath at 55°C for 10 min to release HA from CPC. Any undissolved material was removed through centrifuging the mixture at 8,000 × g for 10 min. Three volumes of ethanol were added to one volume of the resuspended solution to precipitate HA. The purified HA was gathered by centrifuging at 8,000 × g for 10 min, dissolved again in 1 M NaCl solution, and stored at 4°C for further tests. The quantification of HA solution was performed according to the protocol developed by Song, Im, Kang, and Kang (2009). Briefly, 0.5 mL of purified HA solution was mixed with 0.5 mL acetate buffer (0.2 M sodium acetate-acetic acid buffer containing 0.15 M NaCl) and incubated at 37°C for 10 min. The acetate buffer was used
as a blank control. After the incubation, 1 mL cetyltrimethylammonium bromide (CTAB) reagent (2 % (w/v) NaOH solution containing 0.069 M CTAB) was added. The mixture was thoroughly mixed and measured at OD$_{600\text{nm}}$ against the blank control within 10 min. The concentration of HA solution was calculated based on the HA standard (Sigma-Aldrich, 1.00-1.25 MDa) curve which was also measured according to the above procedures.

The dissolved oxygen (DO) in the culture media was measured by DO probe (OxyFerm FDA 225, Hamilton) which was calibrated at 30°C in the modified M17 medium. The pH values of the culture media were tested by a pH meter (S470, Mettler Toledo). The glucose concentration in the culture media was obtained by using a glucose assay kit (Sigma-Aldrich), and all procedures were performed according to the protocol from the kit.

5.3.5 Characterization of cellulose pellicles

To indicate the presence of HA in the BC pellicle produced through co-culturing, the Fourier transform infrared spectroscopy (FTIR) spectra were collected by a Bruker Vertex V70 (Billerica, MA) equipped with a diamond attenuated total reflection (ATR) accessory. Samples were mounted by a high-pressure clamp, and the spectra were the averages of 32 scans in the range of 400 – 4000 cm$^{-1}$ at a resolution of 4 cm$^{-1}$. The crystallinity of cellulose pellicles was measured by collecting x-ray diffraction (XRD) information using PANalyticalX’Pert Pro multi-purpose diffractometer (Almelo, the Netherlands) with Cu Kα radiation generated at 45 kV and 40 mA. The freeze-dried pellicles were compressed into flat pieces and placed on a quartz sample holder. The data
were collected in a reflection mode, and the $2\theta$ range was 5 – 45° with a step size of 0.026°. MDI Jade Software (Materials Data, Livermore, CA) was used to analyze the diffraction diagrams. The crystallinity of samples was estimated by peak deconvolution method where a pseudo-Voigt function was used to fit the peak shape and area, and a broad peak at around 21.5° was assigned as the amorphous contribution (Thygesen, Oddershede, Lilholt, Thomsen, & Ståhl, 2005). The information of crystal size was calculated by the Scherrer equation (Nieduszynski & Preston, 1970):

$$B_{hkl} = \frac{K\lambda}{\sqrt{(\Delta 2\theta)^2 - (\Delta 2\theta_{inst})^2 \cos \theta}}$$

where $B_{hkl}$ is the average crystalline width of a specific plane ($hkl$ Miller indices); $K$ is the shape factor, and the value of 0.9 was used; $\lambda$ is the wavelength of incident x-rays ($\lambda = 0.15418 \text{ nm}$); $\theta$ is the center degree of the peak; $\Delta 2\theta$ is the full width at half maximum (FWHM) of the reflection peak and $\Delta 2\theta_{inst}$ is the instrumental broadening which is 0.0018 radians for this equipment. The morphology of cellulose samples was measured by a Nova NanoSEM 600 (FEI, Hillsboro, OR) field emission scanning electron microscope (FESEM). Lyophilized samples were sputter-coated by a thin layer of iridium and the electron beam was operated at 5 kV under high vacuum. The histogram of ribbon width was measured by ImageJ software (Schneider, Rasband, & Eliceiri, 2012) through counting a total of 100 ribbons from three independent samples for each experimental condition. The tensile tests of cellulose pellicles produced through co-culturing were measured by an Instron (model 3345, Norwood, MA). The freeze-dried cellulose pellicles were compressed into a flat piece at 500 psi by the Instron and cut into 60 × 10 mm pieces for testing. The film tension clamps were used and the experiments were run at 1.0 mm/min under ambient temperature (25°C). Strain ($\varepsilon$) was calculated by $\Delta L/L_0$ where $L_0$
is the initial length of a sample between clamps, and $\Delta L$ is the increased length from $L_0$. Stress ($\sigma$) was calculated by $F/A$, where $F$ is the force applied in Newtons and $A$ is the cross area measured by width multiplying thickness of a sample. Young’s modulus was calculated through the linear region of Stress/Strain. The water holding capacity of the cellulose pellicles was evaluated through their weight loss percentage at 37°C and 30% relatively humidity. Wet cellulose samples were punched into circles with a diameter of 10 mm. The excess water on the surface of the samples was gently removed with Kimwipes (Kimtech) and the samples were accurately weighted every 20 min. The weight loss percentage was calculated by the equation Weight Loss (%) = $(w_0 - w_t)/w_0 \times 100\%$, where $w_0$ is the initial weight of wet pellicles and $w_t$ is the weight of samples at different times.

### 5.3.6 Statistical analysis

The Student’s t-test ($p < 0.05$ or $p < 0.01$) in Minitab Statistical Software (Release 17.1, University Park, PA) was used to evaluate the significant difference between two results. Five to ten samples were measured under each experimental condition for tensile tests, and all the other data points were replicated three times independently. The error bar in all figures indicated the standard derivation (SD).
5.4 Results and discussion

5.4.1 Cellulose pellicles produced in the two-vessel circulating system

Although the peristaltic pump was circulating the culture media between the two aspirator bottles, a relatively static condition was maintained in the BC side vessel and a BC pellicle was synthesized at the air-liquid interface (Figure 5.2a). Compared with the cellulose pellicle produced under static conditions after 7 days (Figure 5.2b), the one formed in the two-vessel circulating system was thinner in thickness which may be caused by the disturbance introduced through the circulating of culture media. Some multi-shaped cellulose pulps and fibers were also observed in the culture media which resembled the shape of BC produced under agitated conditions (Cheng, Wang, Chen, & Wu, 2002).

Figure 5.2 Images of BC pellicles produced in the two-vessel circulating system (a) and a 250 mL flask under static conditions (b) when monoculture G. hansenii ATCC 23769 in the modified M17 medium for 7 days at 30°C. No additional glucose was added, but the air was supplied at the rate of 0.8 vvm in the two-vessel circulating system.

5.4.2 Growth dynamics during co-culture in the two-vessel circulating system

*L. lactis* API3 was constructed based on the P170 expression system with hasABC genes, which were involved in HA synthesis, from *Streptococcus zooepidemicus*. 
Aeration conditions and energy source supplement such as glucose play a significant role in the biosynthetic capacity of *L. lactis* (Nordkvist, Jensen, & Villadsen, 2003). Prasad et al. (2010) reported a 2-fold higher HA production in recombinant *L. lactis* under 1.0 vvm aeration comparing to static conditions. Chauhan et al. (2014) determined that the HA production by *L. lactis* APJ3 was promoted from 750 ± 40 mg/L to 1200 ± 70 mg/L along with the increasing constant feed rate of glucose from 0.5 g/h to 5.0 g/h. When it came to producing BC/HA nanocomposites through co-culture, a suitable range of HA concentration produced by *L. lactis* APJ3 needed to be controlled. According to the research conducted by Ying Li et al. (2015), who manufactured BC/HA through a solution impregnation method with the HA concentration ranging from 0.5%, 0.2%, 0.1%, to 0.05% (w/v), the BC/HA films immersed in 0.1% HA solution showed the shortest wound healing time while the one in 0.05% HA exhibited the best tissue repair results in the *in vivo* experiments. Considering these results and the objective of synthesizing a BC/HA composite with potential application in the medical field, the constant feed rate of glucose was set to 0.067 g/h, 0.033 g/h, or 0.017 g/h and the aeration rate was maintained at 0.8 vvm in all experiments, which was for oxygen supplement and improving the mixing of culture media.

Under these conditions, the pH of the culture media in the two vessels reached equilibrium quickly and showed almost identical values at different times (Figure 5.3). The DO in the BC side bottle exhibited lower values but the same trends compared to the ones in the air side bottle were observed (Figure 5.3). According to the consumption of DO, *L. lactis* APJ3 was mainly growing within 48 hours while *G. hansenii* ATCC 23769 started to grow and dominated the oxygen consumption after 48 hours. At the end of 7
days, the overall HA concentration in the system, when monoculture *L. lactis* APJ3, decreased from 115.5 ± 2.3 mg/L, 92.9 ± 5.3 mg/L, to 77.3 ± 3.5 mg/L with the declining of constant feed rate of glucose from 0.067 g/h, 0.033 g/h, to 0.017 g/h, respectively (Figure 5.4). The dry weight of 23769 BC and Co BC produced in the system was between 0.36 g/L and 0.66 g/L (Figure 5.4) which was significantly lower than BC synthesized under static conditions, typically above 1.0 g/L (Fang & Catchmark, 2015). The reduction of BC agreed with the previous study by Krystynowicz et al. (2002) who supplied aeration for BC production and the possible explanation was the spontaneous formation of cellulose nonproducing mutants when aerating. However, among all the BC samples, the 23769 BC produced by continuously adding glucose at 0.017 g/h was significantly higher than the others. The improvement of BC dry weight could be attributed to the suitable glucose concentration in the culture media which was below 20 g/L after 120 h (Figure 5.5). The reduction in BC yield associated with higher glucose concentration in the culture medium has been reported and extensively discussed (Hwang, Yang, Hwang, Pyun, & Kim, 1999; Masaoka, Ohe, & Sakota, 1993; Son et al., 2003). The mechanism behind the low BC yield is the production of gluconic acid from glucose that drops the pH of the culture medium to suboptimal levels for cell viability, which is typically below 4.0 for *Gluconacetobacter* strains (Keshk & Sameshima, 2006). The changes in pH values when monoculture *G. hansenii* ATCC 23769 under different constant feed rates of glucose agreed with the mechanism. Specifically, for the 0.017 g/h group, the value of pH was 5.0 at the 120th hour and maintained above 4.0 until the end of 7 days (Figure 5.5) whereas for the 0.067 g/h and 0.033 g/h groups, the values of pH were lower, at around 4.5 at the 120th hour, and declined to 4.0 at the 144th hour (Figure
5.3a and d). The mechanism also explained the low production of Co BC in the 0.067 g/h and 0.033 g/h groups where the values of pH already decreased to around 4.0 at the 120th hour (Figure 5.3c and f) although the glucose concentration for the 0.033 g/h group was maintained below 20 g/L (Figure 5.5f). The low yield of Co BC in the 0.017 g/h group was caused by the insufficient supplement of glucose (Figure 5.5i). These growth dynamics of the two strains suggested that controlling the constant feed rate of glucose at the two stages could harvest more BC/HA nanocomposites with desired amounts of HA incorporated. In other words, applying one constant feed rate of glucose within 48 hours would control the HA concentration in the medium. At the 48th hour, the value of pH was around 5.0 under all three co-culture conditions (Figure 5.5c, f, and i) and then changing the constant feed rate of glucose to another level to maintain the glucose concentration below 20 g/L would result in higher production of BC/HA nanocomposites.
Figure 5.3 Values of dissolved oxygen (DO) and pH in the culture media in the two-vessel circulating system when continuously adding glucose at the rate of 0.067 g/h, 0.033 g/h, and 0.017 g/h for monoculture 23769 (a, d, and g), monoculture APJ3 (b, e, and h), and co-culture 23769 and APJ3 (c, f, and i) respectively (n = 3).
Figure 5.4 The dry weight of 23769 BC and Co BC and HA concentration in the media at each vessel when monoculture *L. lactis* APJ3 with the constant feed rate of glucose at 0.067 g/h, 0.033 g/h, and 0.017 g/h (n = 3).
Figure 5.5 Glucose concentration in the culture media in the two-vessel circulating system when continuously adding glucose at the rate of 0.067 g/h, 0.033 g/h, and 0.017 g/h for monoculture 23769 (a, d, and g), monoculture APJ3 (b, e, and h), and co-culture 23769 and APJ3 (c, f, and i) respectively (n = 3).
5.4.3 Characterization of cellulose pellicles from co-culture

The presence of HA in the BC/HA nanocomposites produced through the two-vessel circulating system was measured by FTIR-ATR (Figure 5.6a). The characteristic peaks of HA at 1645 cm\(^{-1}\) and 1544 cm\(^{-1}\) were contributed by the C=O carboxy amide I and C-N-H bending vibration, respectively, which is in agreement with previous reports (Alkrad, Mrestani, Stroehl, Wartewig, & Neubert, 2003; Y. Li et al., 2014). For all Co BC samples, the peak at 1645 cm\(^{-1}\) was observed which indicated that HA was incorporated into the BC network. However, for all 23769 BC, a peak at 1645 cm\(^{-1}\) with relatively lower intensity compared to their Co BC samples was also presented which should be ascribed to H-O-H angle vibration of minor water residues (Olsson & Salmen, 2004), although all samples were freeze-dried and kept in a desiccator before measuring. The peak at 1544 cm\(^{-1}\) was only observed in the Co BC when adding glucose at 0.067 g/h but not in the other two Co BC samples which could be associated with the lower concentrations of HA in the culture medium resulting in less HA incorporated into the cellulose network.
Figure 5.6 Characterizations of 23769 BC and Co BC produced in the two-vessel circulating system under the constant feed rate of glucose at 0.067 g/h, 0.033 g/h, and 0.017 g/h: (a) FTIR-ATR spectra including purified HA extracted from *L. lactis* APJ3; (b) XRD patterns.

The crystal information of BC/HA was revealed through X-ray diffraction. All the Co BC samples exhibited three main peaks corresponding to the (100), (010), and (110) crystallographic planes which were identical to the 23769 BC (Figure 5.6b). After applying peak deconvolution method through MDI jade software, the crystallinity and crystal sizes of 23769 BC and Co BC samples were calculated and summarized in Table 1. Under the same constant feed rate of glucose, the crystallinity of Co BC showed no significant difference to the 23769 BC. However, the circulating of culture medium at 1.5 mL/min in the system for all samples could affect the crystallinity of BC during its synthesis. Czaja, Romanovicz, and Malcolm Brown (2004) reported that cellulose produced from agitated culture conditions presented a decrease in crystallinity when compared to the one synthesized in stationary culture. For the values of *d*-spacing within
crystals, no significant differences were observed between 23769 BC and Co BC under all three constant rates of glucose supplement. However, with the presence of HA, the Co BC exhibited larger mean values of crystal size in all three main planes compared to the 23769 BC. In particular, significant increases in (100), (010), and/or (110) planes were shown in Co BC when comparing values of crystal size between BC samples under the same constant feed rate of glucose (Table 5.1). The increased crystal size in Co BC without changing d-spacing indicated that HA would impact the co-crystallization process during the synthesis of microfibrils. Unlike pectin or hemicelluloses, including xyloglucan, xylan, and arabinogalactan which would decrease crystal sizes (Gu & Catchmark, 2012) or EPS extracted from Escherichia coli ATCC 35860 (K. Liu & Catchmark, 2018) and mannose-rich EPS, HE-EPS (Fang & Catchmark, 2014), which showed no impact on crystal sizes, HA increased the crystal sizes. de Oliveira et al. (2017) also reported an increase in the crystallite of BC/HA produced by directly adding purified HA into the culture medium.

The increase in crystal size may be a result of the combination of the strength of the interaction between BC and HA, the preference of binding site on the forming cellulose, the concentration of the HA and the conformation of the HA on the surface of the cellulose when bound. The lack of change in BC crystallinity suggests that the HA does not prefer to bind to pre-crystalline cellulose (Fang & Catchmark, 2014) but may prefer more ordered cellulose present in a formed or partially formed microfibril. This is evidenced by the increase in cellulose fiber diameter discussed below (Figure 5.8). HA may promote the bundling of the cellulose microfibrils, but the affinity of HA to the ordered cellulose, and the low concentration of HA present in the medium, may not be
sufficient to substantially coat the cellulose microfibrils. By bringing microfibrils into close proximity, however, the HA may promote co-crystallization resulting in the formation of larger crystals. HA, being a linear polymer, may also bind in a linear fashion along the length of the microfibril allowing the microfibrils to align closely also promoting co-crystallization.

Table 5.1 Crystallinity and crystal sizes of 23769 BC and Co BC produced in the two-vessel circulating system (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>23769 BC 0.067 g/h</th>
<th>Co BC 0.067 g/h</th>
<th>23769 BC 0.033 g/h</th>
<th>Co BC 0.033 g/h</th>
<th>23769 BC 0.017 g/h</th>
<th>Co BC 0.017 g/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystallinity (%)</td>
<td>81.1 ± 0.6</td>
<td>80.0 ± 2.3</td>
<td>77.0 ± 5.3</td>
<td>78.0 ± 0.9</td>
<td>75.6 ± 4.0</td>
<td>73.6 ± 3.7</td>
</tr>
<tr>
<td>d-spacing (Å)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>6.15 ± 0.00</td>
<td>6.14 ± 0.02</td>
<td>6.15 ± 0.01</td>
<td>6.14 ± 0.01</td>
<td>6.05 ± 0.02</td>
<td>6.07 ± 0.02</td>
</tr>
<tr>
<td>010</td>
<td>5.29 ± 0.00</td>
<td>5.28 ± 0.02</td>
<td>5.29 ± 0.00</td>
<td>5.27 ± 0.00</td>
<td>5.23 ± 0.02</td>
<td>5.24 ± 0.01</td>
</tr>
<tr>
<td>110</td>
<td>3.92 ± 0.00</td>
<td>3.92 ± 0.01</td>
<td>3.93 ± 0.00</td>
<td>3.91 ± 0.00</td>
<td>3.90 ± 0.01</td>
<td>3.90 ± 0.01</td>
</tr>
<tr>
<td>Crystal Size (Å)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>49.0 ± 1.0</td>
<td>51.5 ± 0.7*</td>
<td>49.3 ± 0.6</td>
<td>53.5 ± 2.1</td>
<td>49.3 ± 0.6</td>
<td>52.0 ± 1.0*</td>
</tr>
<tr>
<td>010</td>
<td>69.3 ± 2.4</td>
<td>75.5 ± 0.7</td>
<td>69.0 ± 1.0</td>
<td>82.5 ± 5.2*</td>
<td>69.0 ± 2.0</td>
<td>74.0 ± 2.6</td>
</tr>
<tr>
<td>110</td>
<td>58.3 ± 2.1</td>
<td>63.0 ± 0.0</td>
<td>59.0 ± 1.0</td>
<td>64.0 ± 1.4*</td>
<td>58.3 ± 0.6</td>
<td>63.0 ± 1.0**</td>
</tr>
</tbody>
</table>

Note: *, p < 0.05; **, p < 0.01 when comparing values of crystal size between 23769 BC and Co BC under the same constant feed rate of glucose.

The surface morphologies of 23769 BC and Co BC are presented in Figure 5.7.

The 23769 BC (Figure 5.7a, c, and e), as control samples, exhibited a porous cellulose network as previously reported (Chi & Catchmark, 2017). When co-culturing with *L. lactis* APIJ3 in the system, denser network with smaller pore size and larger ribbons were observed in Co BC samples (Figure 5.7b, d, and f) compared with the 23769 BC. With decreasing constant feed rate of glucose from 0.067 g/h to 0.033 g/h or 0.017 g/h, the HA
synthesized by the co-cultured *L. lactis* declined, and the pore size in Co BC showed an increasing trend. The incorporation of HA into the cellulose network suggested that BC/HA could present features of HA including improving cell growth and proliferation and mediating early inflammatory response for wound healing (Xiao et al., 2013). The effects of HA on the assembly of cellulose microfibrils were evaluated with the histogram of ribbon width through counting 100 ribbons from three independent samples in each experimental group (Figure 5.8). The average ribbon width increased from around 60 nm for 23769 BC to over 100 nm for Co BC. Lopes, Riegel-Vidotti, Grein, Tischer, and Faria-Tischer (2014) also observed an increase of average ribbon width for BC/HA when adding HA into the culture medium at the beginning of cultivation. Furthermore, with the presence of HA, the upper limit of ribbon width expanded from 180 nm for 23769 BC to 360 nm for Co BC and more ribbon width was distributed between 20 – 40 nm for Co BC rather than 40 – 60 nm, which was the typical width of 23769 BC. HA exhibited similar effects on the assembly of cellulose microfibrils as the EPS extracted from *E. coli* ATCC 35860 (K. Liu & Catchmark, 2018).
Figure 5.7 FESEM surface images of 23769 BC and Co BC produced in the two-vessel circulating system under the constant feed rate of glucose at 0.067 g/h (a and b), 0.033 g/h (c and d), and 0.017 g/h (e and f) respectively. Scale bar: 10 µm and 2 µm in the insert images.
Figure 5.8 Histograms of ribbon width of 23769 BC and Co BC produced in the two-vessel circulating system under the constant feed rate of glucose at 0.067 g/h (a and b), 0.033 g/h (c and d), and 0.017 g/h (e and f) respectively (total counts = 100 per histogram).
The mechanical properties of Co BC were investigated through tensile tests with measuring 23769 BC as control samples. The Young’s modulus of Co BC produced under 0.067 g/h and 0.033 g/h groups were significantly decreased ($p < 0.01$) compared to the 23769 BC synthesized under the same constant feed rate of glucose respectively (Figure 5.9a). At the same time, the strain at break was significantly increased ($p < 0.01$) to 4.5 ± 0.6 % for Co BC from 2.1 ± 0.2 % for 23769 BC under the 0.067 g/h group while no significant change was observed for BC formed within the 0.033 g/h group (Figure 5.9c). The reduced Young’s modulus of Co BC under 0.067 g/h and 0.033 g/h groups should be ascribed to the higher concentration of HA, compared to the 0.017 g/h examples, that gradually coated the surface of BC microfibrils and inhibited the interactions between cellulose fibrils. The presence of HA would allow microfibrils to slip relative to each other when stress was applied and accordingly exhibited an increase in strain at break (Altaner & Jarvis, 2008; K. Liu & Catchmark, 2018). For all BC samples, the stress at break was in the range between 28 MPa and 44 MPa showing no significant changes (Figure 5.9b). Overall, BC/HA still presented excellent mechanical properties compared to pure HA. The mechanically reinforced nanocomposite, BC/HA, is attractive for potential medical applications including skin repair, wound dressing, and as scaffolds for tissue regeneration (Courtenay, Sharma, & Scott, 2018; Petersen & Gatenholm, 2011).
Figure 5.9 Results of tensile tests for 23769 BC and Co BC under the constant feed rate of glucose at 0.067 g/h, 0.033 g/h, and 0.017 g/h: (a) Young’s modulus; (b) stress at break; (c) strain at break; (d) sample curves; **, p < 0.01 (5 ≤ n ≤ 10).

Wet BC exhibits unique characteristics especially high-water content (up to 99%) (W. Hu, Chen, Yang, Li, & Wang, 2014). The presence of HA in the network binds water molecules improving water retention property. (L. Liu et al., 2011). In the medical application such as wound dressing and skin repair, keeping a local moist environment around the wound would promote the healing or renewing of skin (Kamoun, Kenawy, & Chen, 2017). Thus, the water holding capacity of Co BC was investigated through weight loss analysis (Figure 5.10). The 23769 BC samples under all glucose feeding rates were
almost completely dried after 60 min. When HA was integrated with increasing concentration, extended time was needed for Co BC to become completely dry. The Co BC produced under 0.067 g/h group showed the longest time, 120 min, to lose all the water. The enhanced water holding capacity of Co BC could be associated with the combination of the water retention property of HA and the smaller pore sizes in these nanocomposites compared to the 23769 BC (Figure 5.7).

![Figure 5.10](image)

Figure 5.10 Weight loss analysis of 23769 BC and Co BC produced under the constant feed rate of glucose at 0.067 g/h, 0.033 g/h, and 0.017 g/h (n = 3).

5.5 Conclusions

The BC/HA nanocomposites in the pellicle form were directly synthesized through co-culturing *G. hansenii* ATCC 23769 and *L. lactis* APJ3 in a novel two-vessel circulating system. By changing the constant feed rate of glucose from 0.067 g/h to 0.033
g/h or 0.017 g/h, the concentration of HA produced by the co-cultured *L. lactis* APJ3 was decreased. According to the growth dynamics of the two strains where *L. lactis* APJ3 mainly grew within the first 48 hours and *G. hansenii* ATCC 23769 became active after 48 hours, a two-stage regulation on the constant feed rate of glucose was explored for higher production of BC/HA nanocomposites. The incorporation of HA in the cellulose network was indicated by FTIR-ATR analysis. The presence of HA did not affect the crystallinity of the BC but resulted in increased crystal sizes. The average ribbon width and width distribution also increased for Co BC where ribbons as large as 360 nm were observed. With increasing concentration of HA, the strain at break for the BC/HA nanocomposites produced by constant adding glucose at 0.067g/h were increased 113% compared to the 23769 BC and their water holding capacity was enhanced 100%. These characteristics make BC/HA a promising material for application in the medical field and the two-vessel circulating system provides a new approach to directly producing BC based nanocomposites through co-culture.

### 5.6 Acknowledgments

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5.7 References


Chapter 6

Bacterial cellulose/hyaluronic acid nanocomposites production through co-culturing *Gluconacetobacter hansenii* and *Lactococcus lactis* under different initial pH values of fermentation media

6.1 Abstract

Providing additives into the culture media is a traditional method to manufacture bacterial cellulose (BC) based nanocomposites. This study employed a novel fermentation process, which is to co-culture *Gluconacetobacter hansenii* (*G. hansenii*) with *Lactococcus lactis* (*L. lactis*) under static conditions, to synthesize BC/HA (hyaluronic acid) nanocomposites. The HA concentration produced by *L. lactis* and the dry weight of BC/HA during co-culture were regulated by the initial pH values of culture media. The incorporation of HA into the cellulose network increased the crystal sizes when the initial pH values were at 7.0, 6.2, and 5.5. The strain at break was also increased while Young’s modulus was decreased when comparing BC/HA to pure BC produced under the initial pH values of culture media at 7.0 and 6.2. When the initial pH value was 4.0, the HA concentration in the culture media exhibited the lowest level observed, which was 20.4 ± 2.3 mg/L. The BC/HA composite synthesized under this condition exhibited an improved Young’s modulus of 5029 ± 1743 MPa from 2705 ± 656 MPa associated with the pure BC. The FESEM images showed that the presence of HA dramatically changed the distribution of ribbon width in BC/HA compared to that of pure BC.
6.2 Introduction

Bacterial cellulose (BC) has been widely used in various fields including water filtration, food production, paper making and for biomedical applications due to its porous three-dimensional structure, high crystallinity, high purity, high water content, and excellent biocompatibility (Picheth et al., 2017; Rastogi, Singh, Das, Kundu, & Banerjee, 2018; Reiniati, Hrymak, & Margaritis, 2017). To further expand the applications of BC and the manufacture of BC-based nanocomposites, one of the traditional methods is the in situ modification. Specifically, different additives will be provided in the culture media and the BC-based nanocomposites could be produced during the biosynthesis of cellulose microfibrils (Stumpf, Yang, Zhang, & Cao, 2018). These additives, including carboxymethyl cellulose (CMC), xyloglucan, pectin, chitosan, xanthan gum, sodium alginate, and hyaluronic acid (HA), show effects on the morphology, physical properties or yield of BC (Dayal & Catchmark, 2016; de Oliveira et al., 2017; Gu & Catchmark, 2014; Jozala et al., 2016; Szymanska-Chargot et al., 2017). However, supplying these additives during the production of BC would increase the cost, especially for HA, which is expensive (L. Liu, Liu, Li, Du, & Chen, 2011).

K. Liu and Catchmark (2019a) have designed a two-vessel circulating system to directly synthesize BC/HA in the pellicle form through co-culturing Gluconacetobacter hansenii ATCC 23769 (G. hansenii ATCC 23769) and Lactococcus lactis APJ3 (L. lactis APJ3) where the later strain was responsible for the production of HA. In this system, the growth conditions including levels of dissolved oxygen (OD) and glucose concentration were adjustable and BC/HA composites incorporating different amounts of HA were produced. Compared to the typical static fermentation for BC production, this two-vessel
circulating system requires additional supporting devices to achieve control over growth conditions which might not be desired in some cases. In fact, BC-based nanocomposites with improved properties could also be manufactured under static conditions through co-culturing *G. hansenii* with other strains (K. Liu & Catchmark, 2019b). While the operation is simplified in this case, little controls could be applied to the growth conditions in static culture. Therefore, the concentration of the desired product synthesized by the co-cultured strain is not easily adjustable and the amounts that incorporated into the cellulose network might not be in the suitable range critical for controlling the properties of BC-based nanocomposites. For example, BC/HA produced by immersing in 0.1% HA solution showed the shortest wound healing time while BC/HA immersed in 0.05 % HA solution exhibited best tissue repair results during *in vivo* experiments (Ying Li et al., 2015).

In this study, the co-cultured strain, *L. lactis* APJ3, was constructed based on the P170 expression system. The P170 promoter would be up-regulated as lactic acid accumulated in the culture medium and resulted in the decrease of pH (Jorgensen, Vrang, & Madsen, 2014). In the engineered *L. lactis* APJ3, the hasABC genes following the P170 promoter were encoded for hyaluronan synthase, UDP-glucose dehydrogenase, and UDP-pyrophosphorylase which originated from *Streptococcus zooepidemicus* and are critical enzymes for the synthesis of HA (Chauhan, Badle, Ramachandran, & Jayaraman, 2014). With the reduction in extracellular pH values, *L. lactis* APJ3 should be more efficient in the production of HA. However, *L. lactis* MG1363, the parent of *L. lactis* APJ3, grows optimally at pH around 6.5. Its glycolysis is considerably affected when the extracellular pH is below 5 and the growth will stop at pH around 4 (Andersen et al.,
Therefore, decreasing the extracellular pH would inhibit the growth of *L. lactis* APJ3 and result in a decline of the total biomass. Consequently, the HA concentration during static co-culture can be controlled by changing the initial pH values of the culture media. In addition, *G. hansenii* ATCC 23769 was selected for BC production since it could not secrete any significant amount of water-soluble EPS (Fang & Catchmark, 2015). This characteristic makes the strain an ideal model to study the interactions between cellulose and other materials.

By adjusting the initial pH values of the culture media, the BC/HA nanocomposites were manufactured by co-culturing *G. hansenii* and *L. lactis* under static conditions. Changes in pH values in the culture media, the yield of HA, and the dry weight of BC/HA composites were measured at the end of the fermentation. The incorporation of HA in the cellulose network was tested by FTIR-ATR. The crystal information, morphological properties and mechanical strength of BC/HA nanocomposites were evaluated by XRD analysis, FESEM, and tensile tests, respectively.

### 6.3 Materials and methods

#### 6.3.1 Strains and culture media

*Gluconacetobacter hansenii* ATCC 23769 was from the American Type Culture Collection (ATCC). *Lactococcus lactis* APJ3 was developed by Chauhan et al. (2014) through *Lactococcus lactis* MG1363 and P170-based plasmid pAMJ399 from the Bioneer (Denmark). Standard Hestrin-Schramm(HS) medium was used to prepare the primary culture of *G. hansenii* and *L. lactis* which containing 2% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.27% (w/v) sodium phosphate dibasic, 0.115% (w/v) citric
acid, and 0.1% (w/v) magnesium sulfate with the pH adjusted to 5.0 by hydrochloric acid
(Hestrin & Schramm, 1954). The HS agar medium was prepared with an additional 1.5 %
(w/v) agar and erythromycin (Sigma-Aldrich) with a final concentration at 2 µg/mL in
the standard HS medium. The co-culture of *G. hansenii* and *L. lactis* was conducted in
modified M17 medium containing 3% (w/v) glucose, 0.25% (w/v) casein tryptone, 0.25%
(w/v) bacterial peptone, 0.5% (w/v) soya peptone, 0.25% (w/v) yeast extract, 0.5% (w/v)
beef extract, 0.05% (w/v) ascorbic acid, 0.022% (w/v) magnesium sulfate, 1.91% (w/v)
sodium-β-glycerophosphate, 0.35% (w/v) citric acid, and 0.897% (w/v) sodium
phosphate dibasic. The pH values of modified M17 media was tested by a pH meter
(S470, Mettler Toledo) and adjusted to 7.0, 6.2, 5.5, 4.8, and 4.0 with hydrochloric acid
and sodium hydroxide after autoclaving. All the chemicals used in this research were
A.C.S. grade and prepared in ultra-pure water with a resistivity of 18.2 MΩ/cm
(Millipore Milli-Q UF Plus).

### 6.3.2 Co-culture conditions and purification of cellulose pellicles

The colony forming unit (CFU) ratio between *G. hansenii* and *L. lactis* was set as
1:1 at the time of inoculating when co-culturing. The primary culture of each strain was
freshly made each time before co-culturing to maintain the 1:1 ratio for all experimental
groups. For *G. hansenii*, 30 mL HS media, 100 µL thawed frozen stock, and 100 µL
cellulase (Sigma-Aldrich) were mixed and shaken at 250 rpm and 30°C until the optical
density (OD) at the wavelength of 600 nm reached 0.90. An equal volume of fresh HS
medium was used to wash the strains and remove the cellulase. The procedure of washing
was repeated three times, and the centrifuging conditions used for the collection of strains
were 4,500 × g, 4°C, and 5 min. For *L. lactis*, its thawed frozen stock was firstly inoculated on HS agar plates with 2 μg/mL erythromycin to select strains containing the transferred plasmid. The plates were placed in the incubator at 30°C for 24 to 36 hours until clear colonies were formed. Randomly selected colonies on the agar plates were picked and inoculated into 5 mL fresh HS medium containing 2 μg/mL erythromycin and incubated overnight at 250 rpm and 30°C. Another 30 mL fresh HS medium containing 2 μg/mL erythromycin was inoculated with the overnight incubated medium at 1% (v/v) and shaken at 250 rpm and 30°C until the value of OD$_{600\text{nm}}$ reached 0.661 where the strains were in their early log phase. When co-culturing, 1 mL primary culture of *G. hansenii* and 1 mL of *L. lactis* were inoculated into 100 mL modified M17 medium containing 2 μg/mL erythromycin in a 250 mL Erlenmeyer glass flask. After incubating under static conditions at 30°C for 7 days, the cellulose pellicles were collected and washed twice with 0.1 M NaOH solution for 1 hour at 80°C each time. The alkali treated pellicles were rinsed with deionized (DI) water until pH 7.0 was reached. The purified cellulose pellicles were lyophilized (Freezoom 2.5 L, Labconco), accurately weighted (AE240, Mettler Toledo), and stored in a desiccator for further tests. The cellulose synthesized through co-culturing *G. hansenii* and *L. lactis* was labeled as Co BC, and the one for control purpose from monoculture *G. hansenii* was labeled as 23769 BC. Monoculture of *L. lactis* APJ3 was simultaneously conducted under the same culture conditions.
6.3.3 Hyaluronic acid and biomass measurement

The hyaluronic acid produced by monoculture *L. lactis* APJ3 under different initial pH values of culture media was purified and measured based on the methods described in the previous study (K. Liu & Catchmark, 2019a). The biomass of *L. lactis* APJ3 at the end of 7 days was collected by centrifuging at 4,500 × g, 4°C, and 5 min. The resulted pellet was washed twice with 0.9% NaCl solution to remove chemicals attached to the strains. The purified biomass was dried in the oven at 80°C until a constant weight was achieved. The yield of hyaluronic acid (Yp/S) was calculated with the equation Yp/S = dry weight of HA/dry weight of biomass, where the dry weights of HA and biomass were from the same flask for the monoculture of *L. lactis* APJ3.

6.3.4 Fourier transform infrared spectroscopy (FTIR)

The FTIR analysis of purified HA, 23769 BC, and Co BC were tested through a Bruker Vertex V70 (Billerica, MA) equipped with a diamond attenuated total reflection (ATR) accessory. Samples were mounted by a high-pressure clamp, and the spectra were averages of 32 scans in the range of 400 – 4000 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\).

6.3.5 X-ray diffraction (XRD)

The crystalline information of 23769 BC and Co BC was measured by using PANalyticalX’Pert Pro multi-purpose diffractometer (Almelo, the Netherlands) with Cu Kα radiation generated at 45 kV and 40 mA. The freeze-dried BC samples were pressed
into flat pieces and mounted onto a quartz sample holder. The data was generated in reflection mode and collected in the $2\theta$ range of $5 - 40^\circ$ with a step size of 0.026$^\circ$.

MDI Jade Software (Materials Data, Livermore, CA) was utilized to analyze the diffraction patterns. The crystallinity of samples was calculated by peak deconvolution method where a pseudo-Voigt function was used to fit the peak shape and area and a broad peak at around 21.5$^\circ$ was assigned as the amorphous contribution (Thygesen, Oddershede, Lilholt, Thomsen, & Ståhl, 2005). The ratio between the area of all crystalline peaks and the total area of all peaks is the crystallinity.

The information of crystal size was calculated by the Scherrer equation (Nieduszynski & Preston, 1970) with the fitting results:

$$B_{hkl} = \frac{K\lambda}{\sqrt{(\Delta 2\theta)^2 - (\Delta 2\theta_{inst})^2 \cos \theta}}$$

where $B_{hkl}$ is the average crystalline width of a specific plane ($hkl$ Miller indices); $K$ is the shape factor, and the value of 0.9 was used; $\lambda$ is the wavelength of incident X-rays ($\lambda = 0.15418$ nm); $\theta$ is the center degree of the peak; $\Delta 2\theta$ is the full width at half maximum (FWHM) of the reflection peak and $\Delta 2\theta_{inst}$ is the instrumental broadening which is 0.0018 radians for this equipment.

### 6.3.6 Field emission scanning electron microscope (FESEM)

Freeze-dried 23769 BC and Co BC were sputter coated with a thin layer of iridium to observe the morphology and microstructure. A Nova NanoSEM 600 field emission scanning electron microscope (FEI, Hillsboro, OR) was used with the electron beam operating at 5 kV under high vacuum. The bundle widths of samples were
measured by ImageJ software (Schneider, Rasband, & Eliceiri, 2012) with counting 100 bundles three independent samples under each experimental condition.

6.3.7 Tensile testing

The tensile tests of BC pellicles were measured by an Instron (model 3345, Norwood, MA). The lyophilized cellulose samples were pressed into a flat piece at 2,500 Newtons (N) by the Instron and cut into 20 × 5 mm pieces for tensile testing. The film tension clamps were used, and the experiments were run at 1.0 mm/min under an ambient temperature of 25°C. Strain (ε) was calculated as $\Delta L/L_0$ where $L_0$ is the initial length of a sample between clamps and $\Delta L$ is the increased length from $L_0$. Stress (σ) was calculated by $F/A$ where $F$ is the force applied in Newtons, and $A$ is the area of the cross-section of a sample. Young’s modulus was the ratio between Stress and Strain where the linear region was presented.

6.3.8 Statistical analysis

The significant difference between two results was calculated by student test ($p < 0.05$ or $p < 0.01$) in Minitab Statistical Software (Release 17.1, University Park, PA). The mean value of each data point was measured through three replicates, and standard derivation (SD) was used as the error bar in all figures.
6.4 Results and Discussion

6.4.1 The yield of hyaluronic acid

Under the selected initial pH of culture media including 7.0, 6.2, 5.5, 4.8, and 4.0, the values of pH at 168 h were measured for the monoculture of *L. lactis* APJ3 and *G. hansenii* ATCC 23769 and a co-culture of these two strains (Figure 6.1). The lactic acid produced by *L. lactis* APJ3 and the gluconic acid synthesized by *G. hansenii* ATCC 23769 were the main reasons for the decline of pH values of culture media (Zhong et al., 2013). The drop of pH values was mainly attributed to *L. lactis* APJ3 when its initial culture pH readings were 7.0 and 6.2 while *G. hansenii* ATCC 23769 dominated the processes when the initial pH values were 4.8 and 4.0 (Figure 6.1). This phenomenon adhered to the published results that the optimal pH for the growth of *L. lactis* and *G. hansenii* was around 6.5 and between the range 4.0 – 5.0, respectively (Hwang, Yang, Hwang, Pyun, & Kim, 1999; O’Sullivan & Condon, 1999). When the initial pH of the culture medium was 4.0, no further decrease of pH was observed when monoculture *L. lactis* APJ3, which agreed with the previous report that the growth arrestment of the strain occurred around pH 4 (Andersen et al., 2009).
Figure 6.1 pH values of culture media at 168 h when monoculture *L. lactis* APJ3 and *G. hansenii* ATCC 23769 and co-culture these two strains under different initial pH including 7.0, 6.2, 5.5, 4.8, and 4.0 (n = 3). APJ3: monoculture *L. lactis* APJ3; 23769: monoculture *G. hansenii* ATCC 23769; Co: co-culture *L. lactis* APJ3 and *G. hansenii* ATCC 23769.

The yield of HA when monoculture *L. lactis* APJ3 was calculated and shown in Figure 6.2. With the decrease of initial pH values from 7.0 to 4.0, the HA concentration in the culture media reduced from 59.3 ± 0.8 mg/L to 20.4 ± 2.3 mg/L. When the initial pH values were 5.5 and 4.8, the overall HA concentrations were both around 30 mg/L. The Yp/S exhibited a significant increase (*p* < 0.01) when the initial pH values were 4.8 and 4.0 compared to the ones where the initial pH values were 7.0, 6.2, and 5.5. The up-regulated of P170 promoter below the pH 5.5 should be ascribed to the improved Yp/S which was confirmed previously (K. Liu et al., 2014; Madsen, Hindre, Le Pennec,
Israelsen, & Dufour, 2005). The biomass of *L. lactis* APJ3 declined along with the reduction of initial pH values of culture media from 7.0, 6.2, 5.5, 4.8, to 4.0 which cohered to its optimal growth pH at around 6.5 (Andersen et al., 2009).

![Figure 6.2](image)

**Figure 6.2** The HA concentration in media, biomass of *L. lactis* APJ3, and Yp/S (yield of HA) at 168 h when monoculture *L. lactis* APJ3 in the culture media with the initial pH values at 7.0, 6.2, 5.5, 4.8, and 4.0 (n = 3).

### 6.4.2 Production of 23769 BC and Co BC

As previous reported, *L. lactis* APJ3 could consume all the 30 g/L glucose in the culture medium under agitation conditions (K. Liu & Catchmark, 2019a). However, when the culture conditions changed to static with different initial pH values, at least 13.3 ± 1.2 g/L glucose was left in the medium at 168 h when monoculture *L. lactis* APJ3 (Figure 6.3). When co-culturing *G. hansenii* ATCC 23769 and *L. lactis* APJ3, at least 9.4 ± 3.0
g/L glucose remained in the culture medium at 168 h (Figure 6.3) which indicated that glucose was sufficient for the production of HA and BC during the fermentation for all experimental groups. The dry weight of 23769 BC and Co BC is presented in Figure 5.4. When the initial pH of the culture medium was 7.0, the dry weight of both 23769 BC and Co BC was below 0.4 g/L which was significantly lower than BC produced under other initial pH values. These results agreed with the changes in pH values (Figure 6.1) where they were above the optimal range for the growth of *G. hansenii*. No significant differences of dry weight for 23769 BC were observed when the initial pH values declined to 6.2, 5.5, 4.8, and 4.0. The dry weights of these 23769 BC were all above 1.3 g/L. With the presence of *L. lactis* APJ3, the dry weights of Co BC restored to the level of the 23769 BC when the initial pH values reduced from 6.2, 5.5, and 4.8 to 4.0 (Figure 6.4). Since these initial pH values were below the optimal growth pH for *L. lactis* APJ3 and reduced biomass was observed when the pH values decreased from 6.2 to 4.0 (Figure 6.2), *L. lactis* APJ3 should be less active. These combined data suggested that the presence of *L. lactis* APJ3 was not beneficial for the production of BC nanocomposites under static conditions. This is in contrast to similar studies where co-culture was performed between *Gluconacetobacter xylinus* and *Lactobacillus mali* (Seto et al., 2006) or *G. hansenii* and *Escherichia coli* (K. Liu & Catchmark, 2019b). In these cases, the production of BC was significantly enhanced with the presence of the co-cultured strains.
Figure 6.3 Glucose concentration in the culture media at 168 h under different initial pH values including 7.0, 6.2, 5.5, 4.8, and 4.0. The measured glucose concentration in the culture media at the time of inoculation was 28.2 ± 0.7 g/L. The glucose assay kit (Sigma-Aldrich) was used to test the concentration of glucose (n = 3). APJ3: monoculture *L. lactis* APJ3; 23769: monoculture *G. hansenii* ATCC 23769; Co: co-culture *L. lactis* APJ3 and *G. hansenii* ATCC 23769.
Figure 6.4 The dry weight of 23769 BC and Co BC at 168 h produced in the culture media with the initial pH values at 7.0, 6.2, 5.5, 4.8, and 4.0 (n = 3).

### 6.4.3 Fourier transform infrared spectroscopy

The incorporation of HA in the cellulose three-dimensional network was examined using FTIR-ATR (Figure 6.5). The characteristic peaks of HA at 1645 cm\(^{-1}\) was ascribed to C=O carboxy amide I (Alkrad, Mrestani, Stroehl, Wartewig, & Neubert, 2003). For all Co BC samples, the peak at 1645 cm\(^{-1}\) was shown although the peak intensity was weak due to the HA concentrations in the culture media measuring only between 59.3 ± 0.8 mg/L to 20.4 ± 2.3 mg/L. For all 23769 BC, a peak at 1645 cm\(^{-1}\) showed relatively lower intensity compared to their Co BC samples. This peak in 23769 BC should be attributed to the H-O-H angle vibration of minor water residues (Olsson & Salmen, 2004) even though all samples were lyophilized and stored in a desiccator until testing.
Figure 6.5 FTIR-ATR spectra of purified HA, 23769 BC and Co BC produced in the culture media with the initial pH values at 7.0, 6.2, 5.5, 4.8, and 4.0.

6.4.4 X-ray diffraction

By X-ray diffraction, the crystal characteristics of BC/HA were examined. The three main characteristic peaks which corresponded to the (100), (010), and (110) crystallographic planes were all observed in the Co BC synthesized under different initial pH values including 7.0, 6.2, 5.5, 4.8, and 4.0 (Figure 6.6). The crystallinity and crystal sizes of 23769 BC and Co BC were calculated by using peak deconvolution method with MDI jade software and summarized in Table 6.1. With decreasing initial pH values from 7.0 to 4.0, the HA concentration in the culture media was reduced while an increase in the mean value of crystallinity of Co BC was exhibited. Specifically, the crystallinity of Co BC pH 7.0 was $69.1 \pm 3.2\%$ which showed a significant decline compared to the one of 23769 BC pH 7.0 and the Co BC pH 4.8 and Co BC pH 4.0 exhibited comparable
crystallinity values to the ones of 23769 BC pH 4.8 and 23769 BC pH 4.0 which were all around 85%. No significant changes of \(d\)-spacing values were observed when comparing 23769 BC and Co BC synthesized at the same initial pH values of culture media. For the crystal sizes, larger mean values were shown in Co BC compared to the ones of 23769 BC produced under the initial pH values at 7.0, 6.2, and 5.5. Furthermore, a significant increase of crystal sizes in the plane (110) was exhibited in Co BC pH 7.0 and Co BC pH 5.5 compared to the ones of 23769 BC at the same initial pH values. Another significantly larger crystal size in the plane (100) was shown in Co BC pH 6.2 compared to the one of 23769 BC pH 6.2. The increased crystal sizes in BC with the presence of HA was also reported by K. Liu and Catchmark (2019a) and de Oliveira et al. (2017). This increase of crystal size in Co BC without modifying \(d\)-spacing suggested that HA could affect the co-crystallization process during the assembly of microfibrils (Fang & Catchmark, 2014; K. Liu & Catchmark, 2018). However, HA did not increase the crystal size in Co BC pH 4.0 which could be explained by its low concentration produced under the initial pH value of 4.0.
Figure 6.6 XRD patterns of 23769 BC and Co BC produced in the culture media with the initial pH values at 7.0, 6.2, 5.5, 4.8, and 4.0.
Table 6.1 Crystallinity and crystal sizes of 23769 BC and Co BC produced in the culture media with the initial pH values at 7.0, 6.2, 5.5, 4.8, and 4.0 (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>23769 BC pH 7.0</th>
<th>23769 BC pH 6.2</th>
<th>23769 BC pH 5.5</th>
<th>23769 BC pH 4.8</th>
<th>Co BC pH 7.0</th>
<th>Co BC pH 6.2</th>
<th>Co BC pH 5.5</th>
<th>Co BC pH 4.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystallinity (%)</td>
<td>82.0 ± 1.3</td>
<td>85.1 ± 1.9</td>
<td>86.3 ± 0.7</td>
<td>85.3 ± 0.0</td>
<td>69.1 ± 3.2*</td>
<td>73.4 ± 4.7</td>
<td>80.9 ± 0.4</td>
<td>85.1 ± 0.8</td>
</tr>
<tr>
<td>d-spacing (Å)</td>
<td>6.10 ± 0.01</td>
<td>6.12 ± 0.00</td>
<td>6.09 ± 0.01</td>
<td>6.14 ± 0.02</td>
<td>6.14 ± 0.02</td>
<td>6.15 ± 0.01</td>
<td>6.07 ± 0.03</td>
<td>6.12 ± 0.02</td>
</tr>
<tr>
<td>100</td>
<td>5.24 ± 0.01</td>
<td>5.26 ± 0.00</td>
<td>5.24 ± 0.01</td>
<td>5.27 ± 0.02</td>
<td>5.28 ± 0.01</td>
<td>5.29 ± 0.00</td>
<td>5.23 ± 0.02</td>
<td>5.27 ± 0.01</td>
</tr>
<tr>
<td>010</td>
<td>3.90 ± 0.01</td>
<td>3.90 ± 0.00</td>
<td>3.90 ± 0.00</td>
<td>3.92 ± 0.01</td>
<td>3.92 ± 0.00</td>
<td>3.92 ± 0.00</td>
<td>3.89 ± 0.01</td>
<td>3.91 ± 0.01</td>
</tr>
<tr>
<td>110</td>
<td>49.0 ± 0.0</td>
<td>50.3 ± 0.6</td>
<td>51.3 ± 0.6</td>
<td>51.0 ± 0.0</td>
<td>49.7 ± 0.6</td>
<td>52.3 ± 0.6*</td>
<td>52.3 ± 0.6</td>
<td>52.5 ± 0.6</td>
</tr>
<tr>
<td>Crystal Size (Å)</td>
<td>70.5 ± 2.1</td>
<td>70.7 ± 1.5</td>
<td>72.3 ± 0.6</td>
<td>74.5 ± 0.7</td>
<td>72.3 ± 0.6</td>
<td>75.7 ± 5.1</td>
<td>75.3 ± 2.1</td>
<td>72.3 ± 4.0</td>
</tr>
<tr>
<td>100</td>
<td>60.0 ± 0.0</td>
<td>59.7 ± 0.6</td>
<td>60.7 ± 0.6</td>
<td>61.5 ± 0.7</td>
<td>61.7 ± 0.6*</td>
<td>64.0 ± 1.7</td>
<td>63.3 ± 0.6**</td>
<td>63.0 ± 1.0</td>
</tr>
<tr>
<td>010</td>
<td>70.5 ± 2.1</td>
<td>72.3 ± 0.6</td>
<td>72.3 ± 0.6</td>
<td>74.5 ± 0.7</td>
<td>72.3 ± 0.6</td>
<td>75.7 ± 5.1</td>
<td>75.3 ± 2.1</td>
<td>72.3 ± 4.0</td>
</tr>
<tr>
<td>110</td>
<td>60.0 ± 0.0</td>
<td>59.7 ± 0.6</td>
<td>60.7 ± 0.6</td>
<td>61.5 ± 0.7</td>
<td>61.7 ± 0.6*</td>
<td>64.0 ± 1.7</td>
<td>63.3 ± 0.6**</td>
<td>63.0 ± 1.0</td>
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</tbody>
</table>

Note: *, p < 0.05; ** p < 0.01 when comparing values between 23769 BC and Co BC under the same initial pH values

6.4.5 Morphology of 23769 BC and Co BC

The surface morphologies of 23769 BC and Co BC were characterized by FESEM (Figure 6.7). With the presence of HA synthesized by the co-cultured strain L. lactis APJ3, smaller pore sizes and denser fibrils in the cellulose network were observed when comparing the Co BC to 23769 BC. When the initial pH values declined from 7.0 to 4.0, which resulted in the lowest HA concentration among all pH conditions, the Co BC pH 4.0 (Figure 6.7j) showed more similar cellulose network to the 23769 BC while
larger ribbons were also exhibited. For all Co BC samples, no apparent accumulation of HA was observed in the pores or on the cellulose fibrils (Figure 6.7b, d, f, h, and j). The histogram distribution of ribbon widths was generated by counting 100 ribbons from three independent samples under each condition (Figure 6.8). The typical ribbon widths of all 23769 BC were in the range between 40 – 60 nm which adhered to the previous reports (Fang & Catchmark, 2015; Gu & Catchmark, 2012). When HA was incorporated into the cellulose network, the average ribbon width of Co BC was improved to the range of 103 – 125 nm while the ones of 23769 BC were in the range of 60 – 73 nm. Lopes, Riegel-Vidotti, Grein, Tischer, and Faria-Tischer (2014) also reported the increase of ribbon width to over 100 nm in BC/HA manufactured by adding HA into the culture medium during the BC biosynthesis. Ribbons larger than 160 nm, which was the upper limit in 23769 BC, were extensively observed in Co BC and could reach the range of 340 – 360 nm. It was hypothesized that HA coated the surface of co-crystallized microfibrils which promoted the bundling process and resulted in the larger ribbon width (Fang & Catchmark, 2014). Besides, more small ribbons in the range of 20 – 40 nm were counted in Co BC compared to 23769 BC (Figure 6.8). K. Liu and Catchmark (2018) published similar effects of exopolysaccharides (EPS) extracted from *E. coli* ATCC 35860 on forming thinner cellulose ribbons. The possible explanation was the excessive accumulation of EPS or HA on the surface of one plane in crystalline microfibrils interrupted the physical aggregation processes and resulted in smaller ribbons.
Figure 6.7 Surface FESEM images of 23769 BC and Co BC produced in the culture media with the initial pH values at 7.0 (a and b), 6.2 (c and d), 5.5 (e and f), 4.8 (g and h), and 4.0 (i and j) respectively. Scale bar: 10 µm and 2 µm in the insert images.
Figure 6.8 Histograms of ribbon width of 23769 BC and Co BC produced under different initial pH values of culture media including 7.0 (a and b), 6.2 (c and d), 5.5 (e and f), 4.8 (g and h), and 4.0 (i and j) respectively (total counts = 100 per histogram).

6.4.6 Tensile properties

When the initial pH value of the culture medium was 7.0, no intact pellicle was formed at the air-medium interface after 7 days, as the low dry weight shown in Figure 5.4 demonstrates. Therefore, the tensile tests were measured for the 23769 BC and Co BC produced in the culture media with the initial pH values at 6.2, 5.5, 4.8, and 4.0 (Figure 6.9). With the incorporation of HA, Young’s modulus of Co BC pH 6.2 and Co BC pH 5.5 were significantly decreased compared to the 23769 BC synthesized in the culture media under the same initial pH values (Figure 6.9a). The reduction of Young’s
modulus of BC nanocomposites had been extensively reported with the presence of other additives during the biosynthesis of cellulose microfibrils including carboxymethylcellulose (CMC), xyloglucan, pectin, alginate, and hyaluronic acid (Cheng, Catchmark, & Demirci, 2009; Gu & Catchmark, 2014; Kanjanamosit, Muangnapoh, & Phisalaphong, 2010; Y. Li, Qing, Zhou, & Yang, 2014). The embedded additives on the surface of microfibrils were believed to disrupt the direct cellulose-cellulose interactions and result in the decrease of the modulus (Fang & Catchmark, 2014). At the same time, the strain at break for Co BC pH 6.2 and Co BC pH 5.5 was significantly improved from around 2.7% for 23769 BC, to 5.9 ± 1.3% and 4.5 ± 0.7%, respectively (Figure 6.9c). The possible explanation was that the presence of HA coated the surface of microfibrils and allowed these fibrils to slip relative to each other when stress was applied (Altaner & Jarvis, 2008). The further decrease of initial pH values would make L. lactis APJ3 secrete less HA into the culture medium. At an initial pH value of 4.0, the Co BC at pH 4.0 exhibited a significant increase in Young’s modulus while the stress at break and strain at break were comparable to the ones of 23769 BC grown at pH 4.0 (Figure 6.9a, b, and c). This abnormal increase in mechanical properties caused by HA was similar to the study conducted by K. Liu and Catchmark (2018). In their research, Young’s modulus of BC nanocomposites was significantly enhanced only when the culture media containing low levels of EPS, such as 4 mg/L and 8 mg/L, which were extracted from E. coli ATCC 53582. Otherwise, the presence of more EPS in the culture media would decrease the Young’s modulus of BC nanocomposites. The hypothesis for these phenomena is that the low concentration of EPS or HA have the benefits of increasing density (Figure 6.7j) and improving the bundling of larger ribbons
(Figure 6.8), but do not interfere with the stronger co-crystallized microfibril-microfibril interactions as much as when the concentrations of EPS or HA were high.

![Figure 6.8](image)

Figures 6.8 Results of tensile tests for 23769 BC and Co BC produced in the culture media with the initial pH values at 6.2, 5.5, 4.8, and 4.0. a, Young’s modulus; b, stress at break; c, strain at break; d, sample curves (5 ≤ n ≤ 10). *, p < 0.05; **, p < 0.01.

6.5 Conclusions

The BC/HA nanocomposites were directly produced through co-culturing G. hansenii ATCC 23769 and L. lactis APJ3 under static conditions. The reduction of initial pH values of the culture media from 7.0 to 6.2, 5.5, 4.8, and 4.0 resulted in less HA synthesized by the co-cultured strain L. lactis APJ3. The dry weight of Co BC at 7 days
produced through co-culturing was improved with the decline of initial pH from 7.0 to 4.0. The Co BC produced in the culture medium under the initial pH value at 4.0 showed comparable dry weight to 23769 BC which was around 1.4 g/L. The presence of HA in the Co BC was proven by FTIR-ATR analysis. The crystal sizes in BC/HA were increased due to the existence of HA in the culture medium when its initial pH values were at 7.0, 6.2, and 5.5. By comparing the ribbon width distribution between 23769 BC and Co BC, the latter exhibited larger mean ribbon width, which was in the range of 103 – 125 nm and more cellulose ribbons were counted within the range of 20 – 40 nm. The BC/HA synthesized under the initial pH values at 6.2 and 5.5 exhibited decreased Young’s modulus but significantly improved strain at break as compared to the ones of 23769 BC. When the initial pH value declined to 4.0, the BC/HA exhibited a significant increase in Young’s modulus while the strain at break and stress at break were comparable to the ones of 23769 BC. By changing the initial pH values of the culture media, the mechanical properties of BC/HA produced through co-culturing could be adjusted.

6.6 Acknowledgments

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Lactococcus lactis APJ3. This work was supported by the USDA National Institute of Food and Agriculture Federal Appropriations under Project PEN04602 and Accession number 1009850.

6.7 References


Chapter 7

Conclusions and recommendations for future research

7.1 Conclusions

The primary objective of this research is to produce BC-based nanocomposites through co-culturing *G. hansenii* with another strain and investigate the effects of the EPS synthesized by the co-cultured strain on the self-assembly process of BC.

The effects of EPS from *E. coli* ATCC 35860 on the mechanical properties of BC nanocomposites were firstly studied before co-culturing *G. hansenii* with *E. coli*. When *E. coli* ATCC 35860 was grown in HS media at 250 rpm under 30 °C, the EPS produced were composed of seven sugars, including glucuronic acid, rhamnose, fucose, mannose, galactose, glucose, and glucosamine. Under the influence of purified EPS in HS media, *G. hansenii* ATCC 35860 could metabolize it and produce another kind of EPS composed of fructose. Also, a minor part of the added EPS from *E. coli* ATCC 35860 was incorporated into the cellulose fibrillar network.

Since both the EPS from *E. coli* ATCC 35860 and another EPS produced by *G. hansenii* ATCC 23769 possess abundant hydroxyl groups to interact with the BC glucan chains, their effects could be considered as one mixture of EPS. The addition of 1000 mg/L purified EPS into the culture media resulted in the interruption of cellulose-cellulose stacking interactions during the physical aggregation of crystal microfibrils, reducing the overall crystallinity without impacting the crystallization or co-crystallization process during cellulose synthesis. When decreasing the concentration of
purified EPS to 4 mg/L or 8 mg/L, a low end which has been seldom reported, Young’s modulus of the two samples was significantly enhanced compared to that of the control BC, while the crystal size was not affected. This provides a new method for maintaining the crystallinity and crystal size of BC while improving the tensile strength.

To directly produce improved BC-based nanocomposites through fermentation without additives, co-culturing *G. hansenii* ATCC 23769 with *E. coli* under static conditions was studied as a novel fermentation process. During co-culture, the mannose-rich EPS synthesized by *E. coli* ATCC 700728 and *E. coli* ATCC 35860 were incorporated into the BC network and affected the aggregation of co-crystallized microfibrils. When co-culturing *G. hansenii* with *E. coli* ATCC 700728, which produced a low concentration of EPS at 3.3 ± 0.7 mg/L, the BC pellicles exhibited improved mechanical properties with an 81.9% increase in Young’s modulus and a 79.3% enhancement in stress at break while maintaining the crystallinity and crystal sizes as that exhibited by the control BC. The further studies in the growth dynamics of the strains revealed that the two co-cultured strains, *G. hansenii* and *E. coli* ATCC 700728, would require 12 to 24 hours less to reach their maximum CFU values during co-culture. The combined metabolic activities by the two strains played a significant role in co-culture and could be the explanation for the faster synthesis of BC pellicles in the first 24 hours and the 10.8% increase in the production of BC by *G. hansenii* through co-culture.

To control the EPS production during co-culture, a novel two-vessel circulating system was developed as an alternative of static fermentation. The BC/HA nanocomposites in the pellicle form were directly synthesized through co-culturing *G. hansenii* ATCC 23769 and *L. lactis* APJ3 in a novel two-vessel circulating system. By
changing the constant feed rate of glucose from 0.067 g/h to 0.033 g/h or 0.017 g/h, the concentration of HA produced by the co-cultured \textit{L. lactis} APJ3 was decreased. According to the growth dynamics of the two strains where \textit{L. lactis} APJ3 mainly grew within the first 48 hours and \textit{G. hansenii} ATCC 23769 became active after 48 hours, a two-stage regulation on the constant feed rate of glucose was explored for higher production of BC/HA nanocomposites. The incorporation of HA in the cellulose network was indicated by FTIR-ATR analysis. The presence of HA did not affect the crystallinity of the BC but resulted in increased crystal sizes. The average ribbon width and width distribution also increased for Co BC where ribbons as large as 360 nm were observed. With increasing concentration of HA, the strain at break for the BC/HA nanocomposites produced by constant adding glucose at 0.067g/h were increased 113% compared to the 23769 BC and their water holding capacity was enhanced 100%. These characteristics make BC/HA a promising material for application in the medical field and the two-vessel circulating system provides a new approach to directly producing BC based nanocomposites through co-culture.

Since the yield of HA by \textit{L. lactis} APJ3 was directly affected by the extracellular pH, the BC/HA nanocomposites were manufactured through co-culturing \textit{G. hansenii} ATCC 23769 and \textit{L. lactis} APJ3 with different initial pH values of culture media under static conditions. The reduction of initial pH values of the culture media from 7.0 to 6.2, 5.5, 4.8, and 4.0 resulted in less HA synthesized by the co-cultured strain \textit{L. lactis} APJ3. The dry weight of Co BC at 7 days produced through co-culturing was improved with the decline of initial pH from 7.0 to 4.0. The Co BC produced in the culture medium under the initial pH value at 4.0 showed comparable dry weight to 23769 BC which was around
1.4 g/L. The presence of HA in the Co BC was proven by FTIR-ATR analysis. The crystal sizes in BC/HA were increased due to the existence of HA in the culture medium when its initial pH values were at 7.0, 6.2, and 5.5. By comparing the ribbon width distribution between 23769 BC and Co BC, the latter exhibited larger mean ribbon width, which was in the range of 103 – 125 nm and more cellulose ribbons were counted within the range of 20 – 40 nm. The BC/HA synthesized under the initial pH values at 6.2 and 5.5 exhibited decreased Young’s modulus but significantly improved strain at break as compared to the ones of 23769 BC. When the initial pH value declined to 4.0, the BC/HA exhibited a significant increase in Young’s modulus while the strain at break and stress at break were comparable to the ones of 23769 BC. By changing the initial pH values of the culture media, the mechanical properties of BC/HA produced through co-culturing could be adjusted.

7.2 Recommendations for future research

7.2.1 Optimization of culture conditions in the two-vessel circulating system

In the two-vessel circulating system, BC/HA nanocomposites incorporated with different amounts of HA could be synthesized by controlling the constant feed rate of glucose during the co-culture. However, *L. lactis* APJ3 was mainly growing within the 48 hours while *G. hansenii* ATCC 23769 became active after 48 hours. Moreover, at the 48th hour, if the glucose concentration is too high, such as larger than 20 g/L, or the pH value of culture medium is too low, for example below 4.5, the production of cellulose would be inhibited. Thus, a two-stage control on the constant feed rate of glucose is proposed. In other words, sufficient amounts of glucose could be supplied within 48
hours to let \textit{L. lactis} APJ3 secrete the desired concentration of HA, and reduce the constant feed rate of glucose to maintain the glucose concentration in culture medium below 20 g/L.

Besides, due to the P170 expression system in \textit{L. lactis} APJ3, another pH controlling device could be coupled into the two-vessel circulating system if the BC/HA nanocomposites incorporated with a high amount of HA are needed. Jorgensen, Vrang, and Madsen (2014) have reported the correlation between the lactate concentration with the specific productivity of secreted \textit{Staphylococcus aureus} nuclease, which regulated by the P170 expression system (Figure 7.1). By controlling the pH value of culture medium constantly at 6.5, the specific productivity could be adjusted between 0 and 1.7 mg/ODL/h when the lactate concentration was below 225 mM.

The pH controlling process could only be applied within 48 h to achieve a high yield of HA. Moreover, during the synthesize of BC after 48 h, the pH controlling could also be added to improve the BC production. Typically, the gluconic acid metabolized by \textit{G. hansenii} can decrease the pH value of culture medium to the suboptimal range for BC production, and later on, \textit{G. hansenii} would consume the gluconic acid in the culture medium to synthesize BC and result in a natural increase of pH values (Hwang, Yang, Hwang, Pyun, & Kim, 1999). Different from the expectations, controlling the pH values of culture medium between 5.0 and 5.5, which is preferable for cell growth and cellulose production, did not enhance the yield of BC. Whereas, the natural pH shift from 4.0 to 5.5 without controlling showed the best yield. The possible explanation is that low pH in the environment could supply stress to the cells for higher BC production. Thus, the possible strategy to apply pH controlling during BC synthesize to achieve a higher
Production is that declining the pH values of culture medium to around 5.0 at the 48th hour, and let the pH shift naturally. However, the optimal pH to be maintained during the first 48 hours for higher HA production and the best initial pH at the 48th hour to be controlled for improved BC synthesis need to be further studied.

![Graph showing lactate concentration and expression from the P170 promoter in batch culture in chemically defined medium with automatic pH control by potassium hydroxide titration. Specific growth rate (open diamonds) and specific productivity of secreted *Staphylococcus aureus* nuclease (filled triangles) are plotted against the logarithmic mean of lactate concentration in each time interval (n = 5) (Jorgensen et al., 2014).](image)

**7.2.2 Aligned BC grown along the air bubbles**

Due to the random movement of strains in the culture medium during the synthesis of cellulose fibrils, the harvested BC pellicles are randomly oriented BC. Comparing with aligned BC, the latter is preferential when using as the construction
scaffolds to induce unidirectional cell alignment in biomedical fields (Luo et al., 2018; Zhou et al., 2015). To manufacture aligned BC, several methods have been reported. For instance, Sano, Rojas, Gatenholm, and Davalos (2010) applied electric fields to control the moving direction of strains during their synthesis of cellulose, and Putra, Kakugo, Furukawa, Gong, and Osada (2008) cultured BC in oxygen-permeable silicone tubes with inner diameter < 8 mm and obtained a tubular BC with uniaxially oriented cellulose fibrils. Besides, Luo et al. (2018) flowed the culture medium along a fixed direction for the preparation of aligned BC.

In the designed two-vessel circulating system, fresh air was supplied into the system through bubbling in one of the bottles (Figure 5.1). When the aeration rate was 0.8 vvm, a constant flow of air bubbles was formed, and a cellulose tube grown along the bubbles was observed (Figure 7.2). The growing of the cellulose will be in two directions: one is against the direction of gravity, and the length of the cellulose tubes will increase along with the growing time, the other direction is horizontal and towards the center of bubbles which result in the smaller inner diameter of tubular BC.

Figure 7.2 Cellulose tubes grown along the air bubbles. Red arrows indicate the tubular BC.
The outside surface of the tubular BC was observed through FESEM (Figure 7.3). Comparing with the randomly oriented BC (Figure 7.3b), the cellulose fibrils are nicely aligned in the tubular BC (Figure 7.3a). The alignment of cellulose fibrils should be ascribed to the fixed movement direction of air bubbles. With the growing of tubular BC, the inner diameter would become smaller and result in the higher velocity of air bubbles. The alignment of cellulose fibrils should also be observed on the inside surface of tubular BC which needs to be further measured.

The air bubbling process provides a new method to produce aligned tubular BC. The influence of air bubbling velocity and the effects of this process on the assembly of cellulose fibrils need to be further studied. The aligned tubular BC would be a promising material to be applied in biomimetic scaffolds for tissue engineering.

Figure 7.3 FESEM images of outside surface of tubular BC and randomly oriented BC pellicles. a, outside surface of the tubular BC; b, the surface of randomly oriented BC pellicles.
7.3 References


Appendix

Tested culture media for the co-culture of *G. hansenii* and *L. lactis*

<table>
<thead>
<tr>
<th>Components (g/L)</th>
<th>HS Modified 01</th>
<th>HS Modified 02</th>
<th>M17 Modified 01</th>
<th>M17 Modified 02</th>
</tr>
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<tr>
<td>Glucose</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Peptone</td>
<td>5</td>
<td>5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5</td>
<td>5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>2.7</td>
<td>2.7</td>
<td>NA</td>
<td>8.97</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.15</td>
<td>1.15</td>
<td>NA</td>
<td>3.5</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>1</td>
<td>1</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>NA</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium-β-glycerol-phosphate</td>
<td>NA</td>
<td>19.1</td>
<td>19.1</td>
<td>19.1</td>
</tr>
<tr>
<td>Peptone from casein</td>
<td>NA</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Soya peptone</td>
<td>NA</td>
<td>NA</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Casein peptone</td>
<td>NA</td>
<td>NA</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Initial pH</td>
<td>5.0</td>
<td>5.0</td>
<td>7.0</td>
<td>6.2</td>
</tr>
<tr>
<td>HA production by <em>L. lactis</em> APJ3 (mg/L)</td>
<td>$16.2 \pm 0.3$</td>
<td>$5.9 \pm 0.2$</td>
<td>$57.5 \pm 2.2$</td>
<td>$72.2 \pm 2.0$</td>
</tr>
<tr>
<td>Growth of <em>G. hansenii</em> ATCC 23769</td>
<td>Well</td>
<td>Well, even thicker BC after 7 days</td>
<td>Well, initial formation of BC ~ 24h late than HS</td>
<td>Well, no late in the initial formation of BC pellicles</td>
</tr>
</tbody>
</table>

Note: NA indicates the component is not added. The tests were conducted in the two-vessel circulating system at 30°C for 7 days without aeration and glucose supplement.
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EDUCATION

<table>
<thead>
<tr>
<th>Institution</th>
<th>Degree</th>
<th>Years</th>
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<td>Ph.D., Agricultural and Biological Engineering</td>
<td>2014 - 2019</td>
</tr>
<tr>
<td>Harbin Institute of Technology</td>
<td>Master of Engineering, Chemical Engineering</td>
<td>2012 - 2014</td>
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<tr>
<td>Harbin Institute of Technology</td>
<td>Bachelor of Engineering, Food Science and Engineering</td>
<td>2008 - 2012</td>
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</table>

PUBLICATIONS & PROCEEDINGS

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