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Study of a Novel Co-culturing Fermentation for Bacterial Cellulose Nanocomposite Production

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ABSTRACT. Bacterial cellulose (BC), due to its high porosity, high tensile strength, biocompatibility and crystal structure, can be used as a value-added product in the pharmaceutical, food, and processing industries. Studies have been conducted aimed at enhancing BC production and physical properties. Among all the existing methods, polysaccharide addition is one of the most effective ways to increase the production and to alter the mechanical properties of BC. Agitated fermentation, compared to a conventional static cultivation method, is also reported to enhance the BC production. However, there is a lack of study regarding combining agitated fermentation and polysaccharide additive. Moreover, it might not be practical to use polysaccharides directly in industrial production due to cost considerations. This study evaluated an agitated fermentation with pure pullulan additives and a co-culturing system in which the BC-producing microorganisms (*Gluconacetobacter hansenii*) grow together with *Aureobasidium pullulans*, a fungus that produces pullulan as exopolysaccharide (EPS). The study included shake-flask studies, fermentation optimization using response surface methodology (RSM). The research has shown a maximum of 4.5-fold enhancement in the production of BC with pure pullulan addition and a maximum of 6-fold increment for Young's modulus with pullulan addition and co-culturing. The study provided a more in-depth understanding of the performance of *G. hansenii* in agitated and co-culture fermentation systems and could lead to further studies aimed at producing better quality BC products for a variety of applications.

Keywords. *Aureobasidium pullulans*, Bacterial cellulose, Biomaterial, Co-culturing, Fermentation, *Gluconacetobacter hansenii*, Nanocomposites, Polysaccharides, Pullulan.

Introduction

Cellulose, a polysaccharide composed of glucose linked by β (1-4) glycosidic bonds, is one of the most abundant biomaterials in the nature. Cellulose has various sources such as vascular plants and microorganisms. The cellulose produced by microorganisms, particularly bacteria strains such as *Gluconacetobacter, Agrobacterium, Rhizobium, and Sarcina*, is called bacterial cellulose (BC) (Ross et al., 1991; Fang and Catchmark, 2015). Due to its high tensile strength, biocompatibility, and ultrafine reticulated structure, BC has a potential to be used for biomedical applications such as wound care, hernia repair, pelvic floor reconstruction, cosmetic surgery, and others (Backdahl et al., 2006; Hsieh et al., 2008; Fang and Catchmark, 2015; Li et al., 2015).

In the industry, BC are produced by fermentation. For the industries utilizing BC as raw materials, BC production is one of the most important consideration since an improved production could reduce the cost. Moreover, many applications of BC require mechanical strength BC, therefore the mechanical properties of BC (tensile and compressive strength, tear resistance, etc.) is also a major concern for the industry.

Since the BC-producing microorganisms are aerobic strains, the most common method for BC production is static fermentation, in which BC pellicles are formed on the surface of the fermentation broth after inoculation. However, static fermentation has the disadvantage of large surface area requirement, high risk of contamination, and uncontrollable fermentation environment (Cheng et al., 2009). Therefore, agitated fermentation, are being studied. Agitated industrial fermentation utilizes bioreactors, thus has the advantage of lower contamination risk, low footprint requirement, and controlled fermentation parameters (Cheng at al., 2009a).

Besides agitated fermentation, another vastly studied method for improving BC production and mechanical properties is polysaccharide additive. It was observed and hypothesized in studies that polysaccharides that contain linear chain structures, such as levan and xylan, could bind to cellulose glucan chains or microfibrils, altering BC co-crystallization (rate-limiting step for BC production), physical aggregation, and bundling behavior, thus enhancing BC production (Cheng, et al., 2011; Fang and Catchmark, 2014; Liu and Catchmark, 2018; Liu and Catchmark, 2019; Liu and Catchmark, 2020). Some other polysaccharides such as agar, xanthan, and acetan, could enhance the viscosity of fermentation broth, protecting BC-producing microorganisms against harmful shear stress generated by agitation, thus help enhance BC production (Chao et al., 2001; Ishida et al., 2003; Cheng et al., 2011).

Although agitated fermentation and polysaccharide additives had been studied separately for BC production, there is a limited amount of studies combining the two methods. Therefore, for this study, pullulan, an α (1-6) linked polymer of maltotriose (α (1-4) linked glucose trisaccharide), was used as polysaccharide additive, and the effect of pullulan in various concentrations on BC production and mechanical properties was studies in agitated fermentations. The reason pullulan was chosen is that pullulan had been certified as “GRAS” (generally recognized as safe) by Food and Drug Administration (2002). Therefore, pullulan addition would not limit the application of BC. Moreover, since pullulan is an exopolysaccharide produced by a fungal strain *Aureobasidium pullulans*, a novel co-culture fermentation method, in which *Gluconacetobacter hansenii* was inoculated with *Aureobasidium pullulans*, was also evaluated in this study. Compared to utilizing pure polysaccharide additives, the co-culture fermentation bypasses the purification step. The microorganisms for co-culturing could be stocked and reused compared to pure polysaccharides that need to be purchased constantly. Therefore, the co-culturing method could provide a more cost-effective way to produce better quality BC products.

Materials and Methods

Microorganisms and medium

Gluconacetobacter hansenii (ATCC 23769) was chosen as BC producer for this study, and A*ureobasidium pullulans* (ATCC 201253) was chosen as pullulan producer for co-culture fermentation. Both strains were obtained from the American Type Culture Collection (Manassas, VA).

To make all of the results comparable, the base medium was kept the same for the shake-flasks, and the medium were optimized in the later phases of this experiment. The base medium is a mix of standard HS medium and optimum medium for the growth of *A.* pullulans, and the medium contained 50 g/L of glucose, 10 g/L of yeast extract, 5 g/L of peptone, 1.2 g/L of citric acid, 2.7 g/L of Na2HPO4, 5 g/L of KH2PO4, 5 g/L of (NH4)2SO4, 1 g/L of NaCl, and 0.5 g/L of MgSO4 * 7H2O. (Hestrin and Schramm, 1954; Cheng et al., 2009b).

To discover the effect of pure pullulan additive on BC production and mechanical properties, pure pullulan was also added into the medium. The pullulan used in this study was produced by *A. pullulans*, purchased from Sangherb Biotech Inc. (Shaanxi, China).

Methodology

Pullulan addition

For the shake-flask fermentations, 250-ml flasks containing 100 ml of base medium were used. After fresh medium were made and distributed into the flasks, 1, 1.5, and 2% of pure pullulan were added into the flasks. According to the preliminary experiments, *A. pullulans* produces approximately 0.36% pullulan in the base medium under shake-flask condition. To simulate the co-culture fermentation, another group was added with 0.36% pullulan added. Each of the groups was triplicated.

Fermentation

For inoculation, *G. hansenii* stock cultures were inoculated into flasks containing sterilized medium (1% v/v), and incubated statically at 30°C for 3 days. After 3 days of activation, 3% (v/v) of cellulase (Sigma Aldrich, St. Louis, MO) was added into the broth, and the flasks were incubated overnight at 150 rpm, 30°C. Then, the cells were collected by centrifuging (2,500 x *g*, 5 min). After collection, the cells were re-suspended in sterile fresh medium. The collection-resuspension process was repeated for 3 times to wash off the cellulase. After the washing step, the collected cells were re-suspended in 20 ml of sterile fresh medium as the inoculum.

For fermentation, the inoculum (1% v/v) was inoculated into flasks containing sterile fresh medium, and the flasks were incubated at 200 rpm, 30°C for 7-days. After 7 days, BC were collected by filtration using gauze cloth and soaked into 0.1 M NaOH in 80°C water bath for 24 hours to get rid of the cell bodies entrapped. After 24 hours, the BC pellicles were washed using DI water until the pH is neutralized. The washed BC pellicles were then freeze-dried for further analysis. Prior to freeze-drying, the pellicles were shaped in square forms using PVC molds (1-inch x 1-inch). The BC pellicles were than frozen at -80°C overnight and dried by a freeze-dryer after (Freezone 18, Labconco, Kansas City, MO).

Co-culturing

For co-culture fermentation, *G. hansenii* inoculums were prepared with the same method. *A. pullulans* inoculums were prepared by inoculating the stock culture (1% v/v, OD_{600}=2.0 for *G. hansenii*; 1.0 for *A. pullulans*) into flasks containing the medium and incubating at 200 rpm, 30°C for 3 days. For the fermentation, both inoculums were inoculated into the flasks at the same time, and the flasks were also incubated at 200 rpm, 30°C for 7 days. The collection and washing methods were the same as described previously.

Crystallinity, crystal size, and d-spacing

To analyze the crystallinity, crystal size, and d-spacing of BC microfibril planes produced, X-ray diffraction (XRD) was used (PANalytical X'Pert Pro MPD, Malvern Panalitical Ltd., Malvern, United Kingdom). The XRD intensity data were collected in the range of 2θ from 5-30°. The collected intensity data were analyzed using PeakFit software (Systat Sofware Inc., San Jose, CA). The crystallinity was calculated based on the peak integration using pseudo-voight peak deconvolution:

$$\text{crystallinity} = \frac{\Sigma\, A(\text{All the peaks without amorphous})}{\Sigma\, A(\text{All the peaks})} \qquad (1)$$

The crystal size on different crystal planes were calculated using the Scherrer equation (Nieduszynski and Preston, 1970):

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

Where:
B_{hkl} = crystal size on the plane
K = shape factor (a constant related to the method of taking the width of peaks, 0.89 < K <1)
λ = wavelength of X-ray used (1.54059 Å)
Δ2θ = full width half maximum (FWHM) of the peak measured
$\Delta 2\theta_{inst}$ = instrumental width, which is a constant value (0.0018 radians for the method used)

The d-spacing on the crystal planes were calculated based on Bragg's law:

$$d = \frac{\lambda}{2\sin\theta} \qquad (3)$$

Where:
d = d-spacing
λ = wavelength of X-ray used (1.54059 Å)
θ = scattering angle

Bundling and microfibril ribbon widths

The bundling behavior and ribbon widths of BC microfibrils were analyzed visually using scanning electron microscopy (SEM). An Apreo SEM was used for this study (Thermo Fisher Scientific, Waltham, MA). The BC samples were first covered by 10-nm of iridium coating, and then put under the SEM beam. After the SEM images were shot, the bundling behaviors were observed, and the ribbon widths were directly measured based on the images taken. For the measurement, 50 microfibril ribbons were picked randomly from the images, and the width of the ribbons were recorded. Frequency charts of the BC ribbon widths at different conditions were generated and compared.

Mechanical properties

The Young's modulus was used as a representation of mechanical behavior. The mechanical data were measured using a dynamic mechanical analyzer, DMA 800 (Texas Instruments, Dallas, TX). The freeze-dried BC samples were pressed into thin sheets using an Instron mechanical analyzer (Instron, Boston, MA) and cut into smaller pieces with 2-3 mm width and 10-14 mm length. For the test, the width and thickness of the individual samples were first measured by calipers. The samples were then mounted into a tension clamp with 0.05 N of preload force. After installation, the DMA measured the lengths of the sample to calculate the differential strain. The samples were then elongated at a rate of 0.25% strain per minute until broken, and the force applied for the elongation was recorded. The stress was also calculated based on the width and thickness of the samples:

$$S = \frac{F}{W * D} \qquad (4)$$

Where:
S = stress on the cross-section of the sample
F = applied force
W = width of the sample
D = thickness of the sample

The strain (% elongation) was also calculated by ΔL/Lo, where ΔL is the length of elongation, and Lo is the original length of the samples. The stresses vs. strain plot displays a positive linear relationship at the beginning, representing an elastic deformation, and Young's modulus was calculated using the elastic elongation region (linear region) of the stress vs. strain plot.

Fermentation broth analysis

To further understand the behavior of the microorganisms during the fermentation, the fermentation broth was also analyzed. For shake-flask fermentations, the broth was collected after 7-days fermentation, and analyzed for glucose concentration, pH, and viscosity.

The glucose left in the broth was measured using a YSI 2700 Series Biochemistry Analyzer (Xylem Inc., Rye Brook, NY). The analyzer utilizes a glucose-oxidase membrane to oxidize glucose in the media into gluconolactone and hydrogen peroxide. The hydrogen peroxide was then oxidized again and analyzed by sensors (Schlueter et al., 2017).

The pH and the viscosity of the broth was measured using a pH meter (SevenExcellence, Mettler Toledo International Inc., Columbus, OH) and a rheometer (HR-3 hybrid rheometer, TA Instruments, New Castle, DE).

Co-culture fermentation optimization

For the co-culture fermentation, since this is the first study co-culturing *G. hansenii* with a fungal strain, it is unknown that if the base medium is optimum for co-culturing, therefore, the medium composition was optimized for the optimum BC production in this study. Three nutrients were hypothesized to have the most significant effect on BC production: glucose, yeast extract, and peptone (Seo et al., 2004; Son et al., 2010). Response surface methodology (RSM) was used following a Box-Behnken design. The design included 15 individual shake-flask runs, and the combination of the three factors are shown in table 1. 3-D surfaces of BC production in response to the combination of the three factors were generated based on the result of the 15 runs, and the highest point on the 3-D surface was chosen as the optimum combination of the three factors.

Table 1. Box-Behnken Design for Response Surface Generation.

Run order	Glucose (%) [a]	Yeast Extract (%) [a]	Peptone (%) [a]
1	+	+	0
2	0	0	0
3	0	+	+
4	-	-	0
5	0	0	0
6	-	0	+
7	+	0	-
8	0	-	-
9	0	-	+
10	+	0	+
11	-	0	-
12	0	+	-
13	-	+	0
14	+	-	0
15	0	0	0

[a] + represents high end of the selected range (150 g/L of glucose, 50 g/L of yeast extract, and 10 g/L of peptone); - represents the low end of the selected range (50 g/L of glucose, 20 g/L of yeast extract, and 0 g/L of peptone); 0 represents the middle point (100 g/L of glucose, 35 g/L of glucose, and 5 g/L of peptone).

After the optimum combination was selected, the combination was then validated in shake-flasks using the same fermentation methods mentioned in previous sections.

Results

The shake-flask fermentations were conducted first, followed by the optimization in shake-flasks. The optimum medium was then used as the medium for bioreactor fermentations. The results were shown in the order of the experiments.

Shake-flask fermentations with pullulan additive and co-culture fermentation

The BC production in the base medium with various concentrations of pure pullulan additions are shown in Figure 1. According to the result, *G. hansenii* control produced 0.447 ± 0.056 g/L of BC. At a low concentration of pullulan addition (0.36-1%), no significant increase in average BC production was observed. However, as the concentration of pullulan in the medium increased, BC production was enhanced significantly, and the highest BC production was observed with 2% pullulan addition (1.997 ± 0.131 g/L, p=0.008). For co-culture fermentation, no significant change in BC production was observed compared to the control (Figure 2).

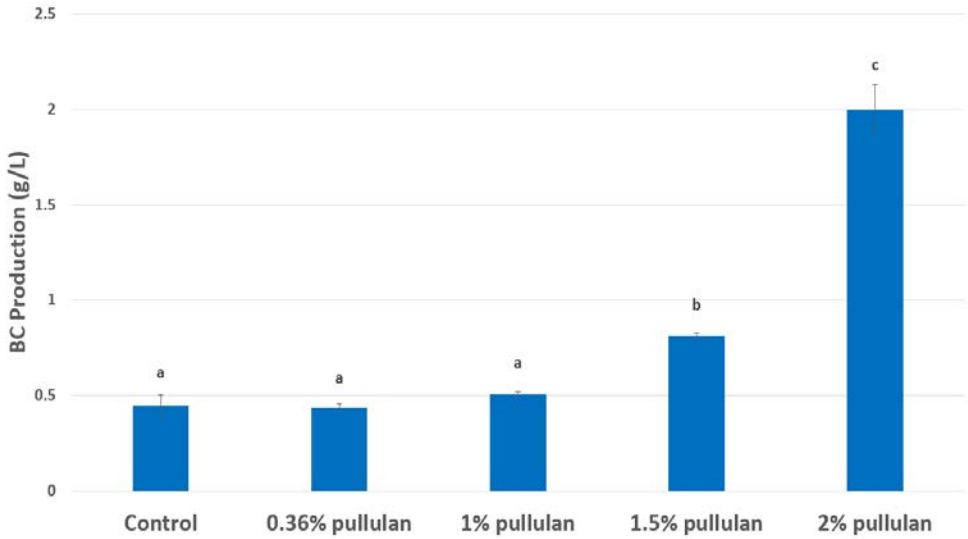

Figure 1. BC production in fermentation medium with various concentrations of pure pullulan additions (n=3, groups in different labels have statistically significant differences, p < 0.05).

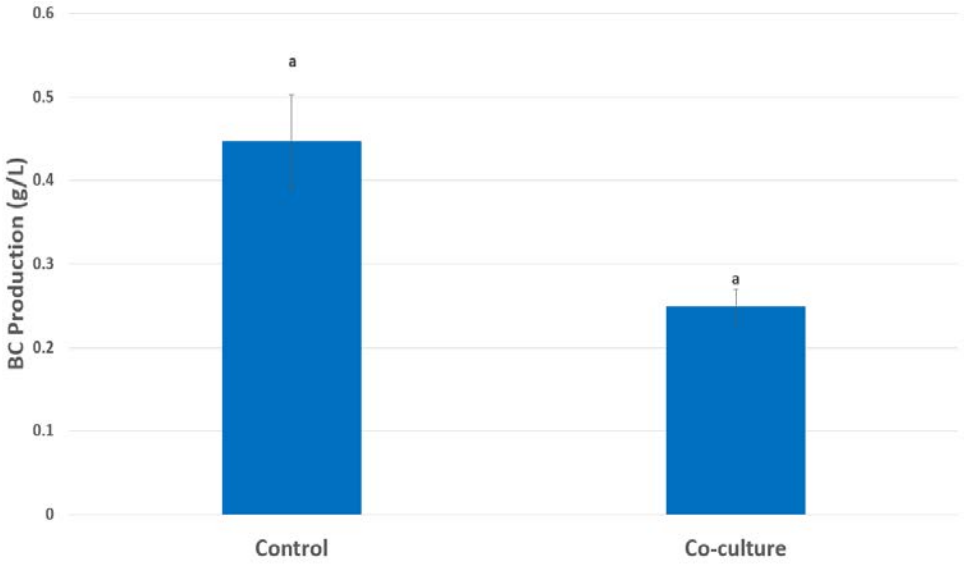

Figure 2. BC production for co-culture fermentation (n=3, groups in different labels have statistically significant differences, p < 0.05).

BC crystallinity, crystal size, and d-spacing

Three major peaks representing the (100), (010), and (110) crystal planes were observed for all of the samples. The XRD intensity patter for co-culture fermentation was also similar to the BC control.

The BC crystallinity, crystal size, and d-spacing on the three major planes were summarized in Table 2. No significant difference in BC crystallinity was observed. The d-spacing for all of the samples were similar except for the BC with 0.36% pullulan supplement, which was hypothesized to be caused by a shifted cellulose type from cellulose I_β to cellulose I_α. The average crystal size on the (110) plane was affected more by pullulan. With 0.36 and 2% pullulan supplement, the average crystal sizes on the plane was reduced significantly ($p<0.05$). However, at 1 and 1.5% pullulan concentration levels, the average crystal size did not have statistically significant reduction. At low pullulan concentration (0.36-1%), the average BC crystal size on the (110) plane was reduced significantly. However, as the pullulan concentration increased to 1.5 and 2%, the crystal size on the (010) plane became similar to the control ($p=0.1740$ and 0.7186, respectively), but the average BC crystal size on the (100) plane was reduced significantly instead. No significant change in crystallinity, crystal size, and d-spacing for co-culture fermentation was observed.

Table 2. Crystallinities, d-spacings, and crystal sizes for BC cultivated with different conditions (n=3, groups with different letters have significant difference, p< 0.05).

	Control	**Co-culture**	**0.36% pullulan**
Crystallinity (%)	81.20 ± 3.24 [a]	80.50 ± 3.08 [a]	86.87 ± 0.53 [a]
d-spacing (Å)			
100	6.08 ± 0.01 [a]	6.07 ± 0.00 [a]	5.97 ± 0.03 [b]
010	5.24 ± 0.00 [a]	5.24 ± 0.00 [a]	5.19 ± 0.02 [b]
110	3.90 ± 0.00 [a]	3.90 ± 0.00 [a]	3.86 ± 0.01 [b]
Crystal size (Å)			
100	52.90 ± 0.41 [a]	53.83 ± 0.22 [a]	51.56 ± 0.41 [a]
010	75.52 ± 4.28 [a]	72.16 ± 1.14 [a]	62.74 ± 0.51 [b]
110	59.33 ± 0.71 [a]	59.19 ± 0.52 [a]	56.56 ± 0.27 [b]
	1% pullulan	**1.5% pullulan**	**2% pullulan**
Crystallinity (%)	88.96 ± 1.14 [a]	79.00 ± 5.25 [a]	81.17 ± 0.76 [a]

d-spacing (Å)			
100	6.03 ± 0.03 [a]	6.04 ± 0.02 [a]	6.06 ± 0.02 [a]
010	5.23 ± 0.01 [a]	5.23 ± 0.02 [a]	5.24 ± 0.02 [a]
110	3.89 ± 0.01 [a]	3.89 ± 0.01 [a]	3.90 ± 0.01 [a]
Crystal size (Å)			
100	51.60 ± 0.26 [a]	51.08 ± 0.45 [b]	51.10 ± 0.12 [b]
010	65.83 ± 1.44 [b]	67.66 ± 2.08 [ab]	64.91 ± 2.34 [b]
110	57.13 ± 0.36 [ab]	57.15 ± 0.34 [ab]	56.54 ± 0.14 [b]

BC bundling and microfibril ribbon widths

50 BC ribbons were picked randomly from the SEM images taken for each of the groups. The result of the measurements had shown that the average BC ribbon width had increased significantly for all of the treatments (including co-culturing) compared to the BC control (Figure 3).

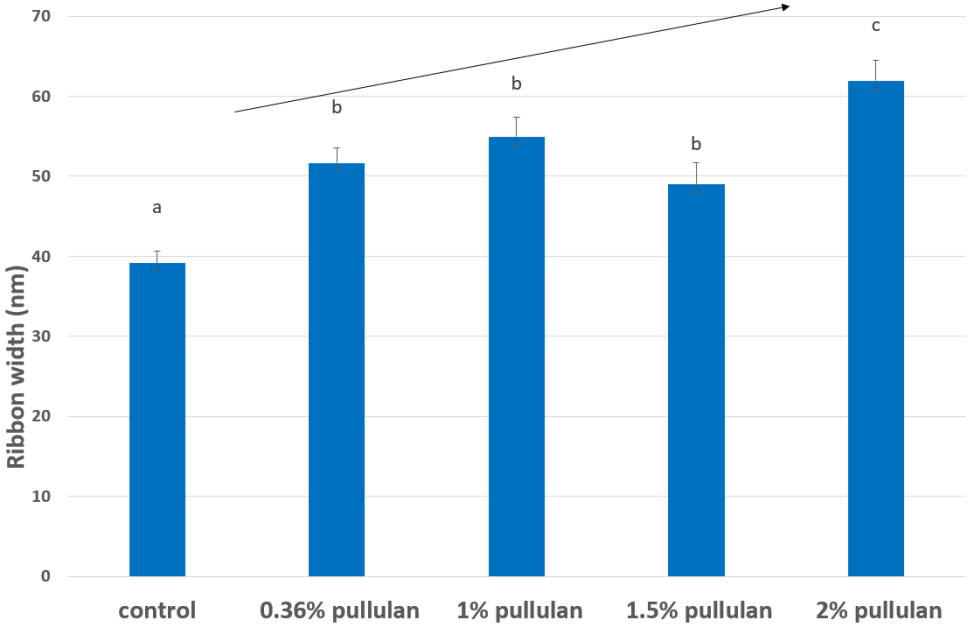

Figure 3. Average BC ribbon widths with different treatments (n=50, groups with different letters have significant differences, $p < 0.05$).

BC mechanical behaviors

BC produced by different treatments all had similar deformation behaviors: in the beginning, the stress the samples endure had a positive linear relationship with the strain, which represented elastic deformation. After the elongation reached approximately 0.5-1% strain, the samples started deforming inelastically until break. The BC samples produced in this study all broke at the maximum stress they endure, therefore, the stress at the break points could be used to represent tensile strength.

The average Young's modulus of BC control is 55.24 ± 14.57 MPa, and Young's modulus of BC tended to increase with pullulan concentration at low level (0-1%), and as the concentration of pure pullulan further increased, the Young's modulus was reduced back to the same level as BC control (Figure 4). The maximum Young's modulus in this study was BC cultivated in medium with 1% pullulan addition (333.77 ± 61.74 MPa). The tensile strength of BC had a positive relationship with Young's moduli. BC produced by co-culturing also had significantly higher Young's modulus compared to the control (161.33 ± 17.90 MPa).

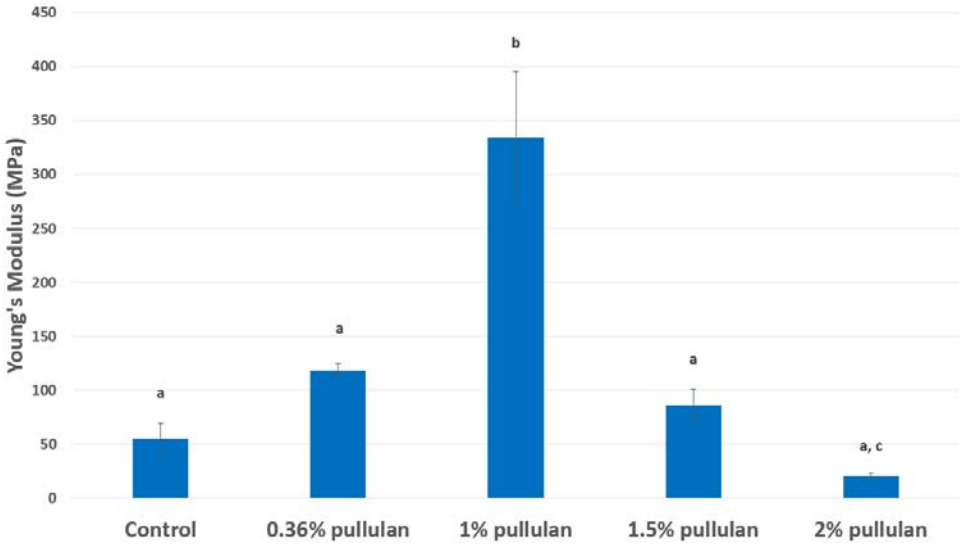

Figure 4. Young's modulus of BC with different treatments (n=3, groups with different letters have significant differences, $p < 0.05$).

Fermentation broth analysis

BC fermentation with pullulan addition did not have significant difference in glucose consumption compared to the control. However, the co-culture fermentation had consumed significantly less glucose compared to the control. The fermentation broth with 1, 1.5, and 2% pullulan addition had a significantly lower final pH. The lower final pH was a representation of a better growth of *G. hansenii* since the microorganism produces acetic acid as a bi-product during the fermentation.

The viscosities of fermentation broth were also measured. The viscosities of fresh medium and the broth after fermentation were measured to determine if *G. hansenii* produced anything that could alter the viscosity of the medium. The broths did not have any significant change in viscosity before and after the fermentation process for pure *G. hansenii* inoculation. Therefore, it was hypothesized in this study that the only cause for an altered viscosity is pullulan, either added into the medium or produced during the co-culture fermentation, and the viscosity of the medium increased proportionally as a result of increased pullulan concentration.

Fermentation optimization

The result of the 15 co-culture fermentation optimization tests (Table 1) were analyzed using Minitab Statistical Software (Version 17, Minitab LLC., State College, PA), and a model representing the response surface was produced by the software. The ANOVA for each of the factors were checked so that all the factors included in the model were statistically significant. The model had an R^2 of 0.9371, the adjusted R^2 was 0.9022, and the predicted R^2 for this model was 0.9030. The equation for the model is shown as equation 5.

$$BC\ production\ \left(\frac{g}{L}\right) = -18.91 + 11.29 * [Glu] - 4.32 * [YE] + 5.17 * [Peptone] - 7.56 * [Glu] * [Glu] + 7.28 * [YE] * [Peptone] \quad (5)$$

Where:
[Glu] = concentration of glucose (% or g/100 ml)
[YE] = concentration of yeast extract (%)
[Peptone] = concentration of peptone (%)

According to the model, the optimum concentration of glucose is approximately 62 g/L. However, BC production in response to the combination of yeast extract and peptone was complex: the co-culture fermentation had better BC production when low concentrations and high concentrations of yeast extract and peptone were combined. Since the observation was abnormal, both of the conditions were validated in shake-flasks, and the conditions were named based on the concentration combination (Low-low and High-high). The medium compositions of the Low-low and the High-high mediums are shown in Table 3. The validation process was conducted in shake-flasks (n=6), and the actual BC production and the glucose consumption for the two optimum conditions are also shown in Table 3.

Table 3. Medium composition, predicted BC production, and actual BC production for the optimum co-culturing conditions.

Ingredient	Low-low	High-high
Glucose (g/L)	62.2	62.7
Peptone (g/L)	0	10
Yeast Extract (g/L)	20	50
Citric Acid (g/L)	1.2	1.2
Na_2HPO_4 (g/L)	2.7	2.7
KH_2PO_4 (g/L)	5	5
$(NH_4)_2SO_4$ (g/L)	5	5
NaCl (g/L)	1	1
$MgSO_4 * 7H_2O$ (g/L)	0.5	0.5
BC production (predicted and 95% CI) (g/L)	0.3478 (0.2592, 0.7786)	0.3901 (0.2703, >0.8)
BC production (actual)(g/L)	0.3048 ± 0.02	0.1852 ± 0.01
Glucose consumption (g/L)	34.19 ± 0.92	28.44 ± 2.85

The predicted interval of the optimum model had a large variation (shown as 95% confidence interval). For the Low-low condition, the average BC production of co-culturing (0.3048 ± 0.02 g/L) was 22.4% higher compared to the original medium (0.249 ± 0.007 g/L). However, the average BC production for the high-high condition was lower than the predicted value and the original medium.

The crystallinity, crystal size, and d-spacing for BC produced in the two optimum conditions are shown in Table 5. No significant change in crystallinity and d-spacing was observed. For crystal size, the BC microfibril crystal size was also lower on the (010) and (110) planes, similar to BC produced in the base medium with low concentration pullulan addition.

Both the Low-low and the High-high condition had larger average BC ribbon width. As for mechanical property, the Young's moduli were also analyzed using DMA (Figure 5). BC produced in both of the medium had significant improvements in Young's modulus. BC produced in the high-high condition had the highest Young's modulus (914.43 ± 117.40 MPa). The Low-low and the High-high conditions had similar result as BC produced in base medium with 0.36-1% pullulan addition. The behavior of BC in the Low-low medium was more similar to the ones BC with 0.36% pullulan addition, and the behavior of BC produced in the High-high medium was closer to BC with 1% pullulan addition.

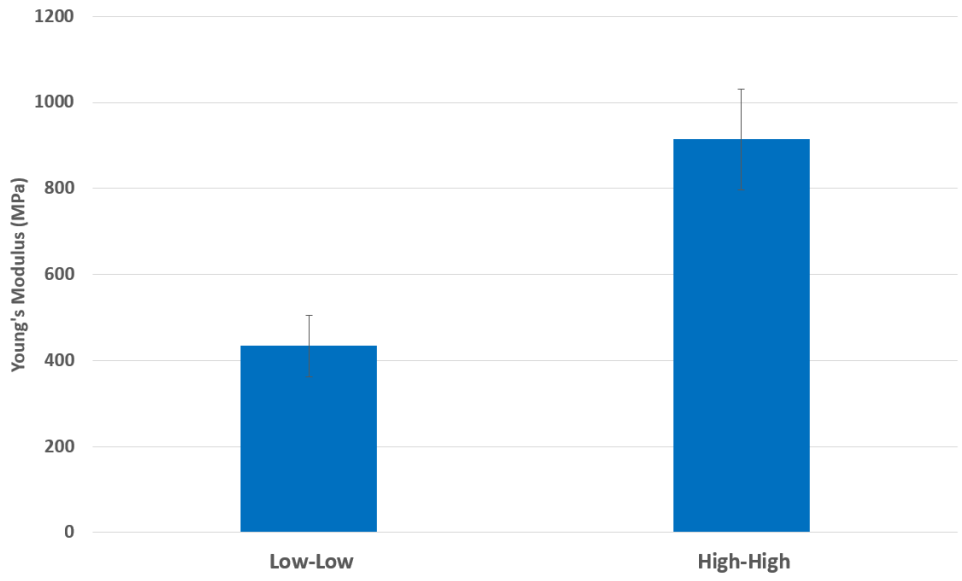

Figure 5. Young's moduli of BC cultivated in the optimum medium (n=3).

Discussions

To understand the effect of pullulan on BC production, the schematic of BC formation needs to be understood first (. At the beginning of BC formation, 10-15 glucan chains inside the cytoplasmic membranes of BC producing microorganisms were gathered and for BC protofibrils with the help of an enzymatic complex, cellulose synthase, and excreted from the

cells. The formation of BC protofibrils is called polymerization. After being excreted, parallel adjacent BC protofibrils co-crystallize to form larger BC microfibrils. The BC microfibrils form large BC ribbons through two major processes: 1) Physical aggregation, in which BC microfibrils join together under the influence of either hydrogen bonding or glucan chain interactions; 2) Bundling, in which BC microfibrils join together with the help of polysaccharides, such as hemicellulose or carboxymethylcellulose (CMC). During BC synthesis, co-crystallization, physical aggregation, and bundling take place at the same time, but with different degrees (Fang and Catchmark, 2014). The effect of pullulan on BC formation could take place in all the processes mentioned.

Effect of pullulan on BC synthesis

With a low concentration of pullulan addition, the crystal size on the (010) and (110) planes were reduced significantly (Table 2) with no significant change in crystallinity. The observation was different with the finding of Fang (2014, 2015) and Liu (2018) where decreased crystallinity and no change in crystal size was observed. However, the observation was consistent with some other studies treating BC with xyloglucan, xylan, and mannan (Atalla et al., 1993; Uhlin et al., 1995; Botten et al., 2008). Therefore, it was hypothesized that the difference between polysaccharides on affecting BC crystallites could be related to the structure of the polysaccharide and the spatial orientation of the binding between the polysaccharide and BC fibers.

Based on the previous studies and the experiment results, it was hypothesized in this study that pullulan, due to the existence of α (1-4) and α (1-6) glycosidic bonds and linear backbones, have a high affinity to BC microfibrils. Similar to many other polysaccharides (Fang and Catchmark, 2015; Liu and Catchmark, 2019), pullulan could have a binding preference to the ordered pre-crystallized BC microfibrils on the (010) and (110) planes. The binding of pullulan could disrupt co-crystallization, leading to smaller crystal sizes on the (010) and (110) planes (Benziman et al., 1980; Cheng et al., 2011; Weng et al., 2019). With a higher concentration of pullulan in the medium (1.5-2%), it was hypothesized that pullulan started saturating the hydrogen bonding sites on the (010) and (110) planes, therefore, a decreased crystal size on the (100) plane was observed (Table 2).

Moreover, as described in the studies conducted by Cheng (2011), Fang (2014), and Liu (2018, 2019, 2020), it was hypothesized that the bundling behavior of BC microfibrils was promoted by pullulan. Therefore, an increased BC ribbon width was observed as the concentration of pullulan in the medium increased. Another possible explanation of pullulan improving BC bundling is that pullulan, as a thickening agent, could increase the viscosity of the medium, making the produced BC endure less shear force generated by agitation. To determine if there is a relationship between the medium viscosity and the BC ribbon with, a dot-plot was also generated. As a result, a relatively weak positive linear relationship was determined (R^2=0.6239), suggesting that the BC ribbon width might have a correlation with the viscosity of the medium, but viscosity might not be the principal factor determining BC ribbon width.

Effect of pullulan and co-culturing on BC production

No significant improvement in BC production was observed in medium with low concentration of pullulan added (0.36-1%). However, as the concentration of pullulan further increased to 1.5-2%, the BC production had been improved significantly. It had been shown in previous studies that the addition of some polysaccharides, such as carboxymethylcellulose (CMC), or other specific non-polymer additives, such as fluorescent dyes (Calcofluor white and Brilliant yellow), could help improve BC production. The hypothesized reason is that the additives could bind to pre-crystallized BC microfibrils, disrupting the co-crystallization process, which is the rate-limiting step for BC production (Benziman et al., 1980; Cheng et al., 2011; Weng et al., 2019). Therefore, it was also hypothesized that in this study, pullulan interacted with BC and disrupted co-crystallization similarly as CMC and the fluorescent dyes did.

Another explanation of pullulan enhancing BC production is that pullulan, as mentioned previously, could increase the viscosity of the fermentation medium reducing the shear force endured by the *G. hansenii* and BC. Therefore, it is possible that pullulan could protect *G. hansenii* against the shear force generated by shaking. Shear stress had been proven to be harmful for BC production (Chao et al., 2001; Ishida et al., 2003; Cheng et al., 2011). The explanation is also consistent with the observation since BC production was enhanced after the pullulan concentration exceeded 1%, and the viscosity of the fermentation broth also started increasing significantly after the pullulan concentration reached to similar level.

For co-culture fermentation, no significant difference in BC production was observed. A possible explanation is that, although pullulan produced by the fermentation could improve BC production, *A. pullulans* and *G. hansenii* might had disrupted the growth of each other since glucose consumption for co-culturing was significantly less than the control. To verify the hypothesis, another co-culture fermentation was conducted, in which the microorganisms were incubated in the base medium with tripled carbon and nitrogen sources (50 g/L of glucose, 30 g/L of yeast extract, 15 g/L of peptone, 1.2 g/L of citric acid, 2.7 g/L of Na_2HPO_4, 5 g/L of KH_2PO_4, 5 g/L of $(NH_4)_2SO_4$, 1 g/L of NaCl, and 0.5 g/L of $MgSO_4 * 7H_2O$). The fermentation still produced significantly less BC after 7 days (0.288 ± 0.015 g/L). Therefore, we hypothesize that the growth competition between *G. hansenii* and *A. pullulans* did exist for the co-culture fermentation, and the competition might be on nutrients other than the carbon and nitrogen sources (salts, minerals, etc.) since tripling the concentrations of them did not improve BC production significantly.

Effect of pullulan and co-culturing on BC mechanical behaviors

The Young's modulus of BC tends to increase when the concentration of pullulan increased from 0 to 1% and decrease after the pullulan concentration exceeded 1%. BC produced in medium with 1% pullulan addition had significantly higher Young's modulus than BC control. We hypothesized that the change in the Young's modulus of BC is related to the density of hydrogen bonds. For BC pellicles, the most essential factor affecting their tensile strength is hydrogen bonds between BC microfibrils, more specifically, between the glucan chains forming the microfibrils (Liu and Catchmark, 2019; Liu and Catchmark, 2020). At a low concentration of pullulan addition (0-1%), pullulan binding to the microfibrils promote BC bundling, increasing the density of hydrogen bonds. More hydrogen bonding result in higher Young's modulus. At a low concentration, pullulan was insufficient to coat the microfibrils thoroughly. However, as the concentration of pullulan in the medium reached 1.5%, pullulan started coating all the microfibrils substantially, disrupting the physical aggregation of BC microfibrils. The disruption on physical aggregation reduces the Young's modulus of BC produced. For the co-culture fermentation, since the pullulan produced was approximately 0.36%, the Young's modulus was similar to BC produced in medium with 0.36% pullulan addition, which was consistent with the ribbon width result.

Co-culture fermentation optimization

For the optimized medium, the crystal size on the (010) and (110) planes were reduced, similar to BC produced in medium with 0.36-1% pullulan addition. Since the average BC ribbon width in the High-high medium was higher than the BC ribbon width in the Low-low medium, we hypothesized that the High-high medium promoted the growth of *A. pullulans* more than the Low-low medium. More pullulan was produced in the High-high medium but the pullulan production did not exceed 1% even in the High-high medium since an increased BC Young's modulus and tensile strength was still observed for the High-high condition. BC production was improved in the Low-low medium compared to co-culturing in the original base medium. However, the BC production was reduced in the High-high medium. The result in the High-high medium was similar to co-culturing in medium with tripled nitrogen sources. A possible explanation is that, as mentioned previously, the growth competition *A. pullulans* and *G. hansenii* exists during co-culturing. Since *A. pullulans* is a fungal strain, it prefers richer medium. Therefore, the Low-low medium could more in favor of the growth of G. hansenii, while higher concentration of nitrogen source made the High-high medium more in favor of the growth for *A. pullulans*, inhibiting the growth and BC production for *G. hansenii*.

Conclusion

In conclusion, this study has demonstrated that an addition of 1.5 and 2% pure pullulan could improve the BC production. The BC production was doubled with 1.5% pullulan addition, and increased for 4.5-fold with 2% pullulan addition. A small amount of pure pullulan addition could increase the Young's modulus and tensile strength of BC, and the highest improvement was observed for BC produced in medium with 1% pure pullulan addition (6-fold increment). Co-culture fermentation, although did not improve BC production, increased the Young's modulus of BC for 3-folds. We hypothesized that a low concentration of pullulan addition and co-culture fermentation could improve the mechanical behaviors of BC by improving the bundling behavior, while a high concentration of pullulan addition could thoroughly coat the BC microfibrils, disrupting co-crystallization, thus improve BC production significantly. Since high concentration of pullulan coated the BC microfibrils, it could disrupt the physical aggregation of BC microfibrils significantly and compromise the mechanical properties of BC pellicles. For co-culturing, we also hypothesized that a growth competition between the two microorganisms inoculated (*A. pullulans* and *G. hansenii*) exists, although the pullulan produced by *A. pullulans* help improved the mechanical behavior of BC Thereforfe, this study had proven that pullulan could alter co-crystallization, bundling and physical aggregation of BC fibers, improving BC production and mechanical properties at different concentrations. The study had also shown that BC pellicles with better physical properties could be produced through co-culturing. Although the mechanisms of co-culture fermentation need to be discovered more thoroughly since co-culturing *G. hansenii* with fungal strains had never been conducted before, this study is a good beginning since it has confirmed the potential of co-culture fermentation as a more economical way to produce better quality BC products in the future.

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