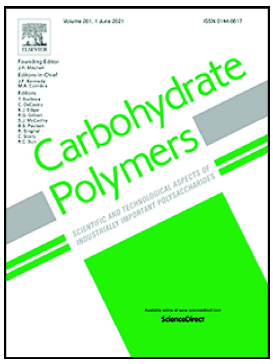

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Biofilm inspired fabrication of functional bacterial cellulose through ex-situ and in-situ approaches

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Abstract

Bacterial cellulose (BC) has been explored for use in a range of applications including tissue engineering and textiles. BC can be produced from waste streams, but sustainable approaches are needed for functionalisation. To this end, BslA, a *B. subtilis* biofilm protein was produced recombinantly with and without a cellulose binding module (CBM) and the cell free extract was used to treat BC either ex-situ, through drip coating or in-situ, by incorporating during fermentation. The results showed that ex-situ modified BC increased the hydrophobicity and water contact angle reached 120°. In-situ experiments led to a BC film morphological change and mechanical testing demonstrated that addition of BslA with CBM resulted in a stronger, more elastic material. This study presents a nature inspired approach to functionalise BC using a biofilm hydrophobin, and we demonstrate that recombinant proteins could be effective and sustainable molecules for functionalisation of BC materials.

Keywords

bacterial cellulose, recombinant BslA, cellulose binding module, in-situ modification, ex-situ modification

1. Introduction

Cellulose is the most abundant organic molecule on earth. It is readily found in nature, and can withstand acids, alkalis and high temperatures. Furthermore, it comes in many forms and is extremely meuble (Cannon & Anderson, 1991; Hassan, Hassan, Moorefield, & Newkome, 2015; Römling & Galperin, 2015). Cellulose is mainly produced by plants; however, bacteria can produce cellulose with higher purity, crystallinity, tensile strength, and a higher degree of water holding capacity. Bacterial cellulose (BC) has been explored in various applications, including for biomedical applications such as artificial skin, chronic wound treatment and as a scaffold for engineered tissue (Cherng et al., 2021; Meng et al., 2019; Zheng, Li, Luo, & Wang, 2020). More recently BC has been highlighted as a potential new material for the textile industry as sustainable fibres or a nonwoven 'bio leather' (Provin et al., 2021).

Like all cellulosic polymers BC has an abundance of hydroxyl bonds to create single cellulose chains, which connect to create microfibrils and ribbon fibrils. BC is produced in a liquid culture where a pellicle forms as the fibril network combines into a mat at the liquid / air interface (Torres, Commeaux, & Troncoso, 2012; Wang, Tavakoli, & Tang, 2019). The hydroxyl groups provide active sites for both physical and chemical reactions and are the basis of BC's high material strength when the material is wet. BC material is highly hydrophilic, with a low water contact angle (WCA) of around 30°. Due to this nature, when the materials are saturated with water for long periods of time, the structure of the cellulose fibres degrade because the hydrogen bonds between fibres are sequestered by water, causing the material to swell and the mechanical strength to decrease (Pirzada, Ashrafi, Xie, & Khan, 2020). The porosity and absorbency of BC are advantageous for some applications, for example, wound dressing and food additives, but limits its applications in other fields such as for filter membranes and textiles (Galdino et al., 2020; Provin et al., 2021). Therefore, the modification of BC materials to increase hydrophobicity, for different applications has attracted research interests from a wide range of disciplines.

Several methods have been proposed using a range of physical and chemical post-production processes to increase hydrophobicity to BC (Provin et al., 2021), however, they have limitations. For example, the physical treatment to increase the BC hydrophobicity through ultrasound (Ybañez & Camacho, 2021) requires high energy input and the chemical modification of cellulose-based materials with functional groups is limited by the choice of limited solvents. Moreover, the utilization of these toxic chemical agents has

serious negative effects on the environment, simultaneously influencing the sustainability of the production methods and adding complexity in waste discharge, thus limiting its large-scale production (Ifuku et al., 2007; Lee et al., 2011; Pertile, Andrade, Alves, & Gama, 2010; Rouabhia et al., 2014; Zhou et al., 2016).

A key advantage of BC production is that it is a biological fabrication process. It is, therefore, energy efficient and leads to materials with complexity in a single production/growth step. To this end we have been investigating biological solutions to the modification of BC properties. Using proteins to modify biomaterial properties to add functionality has been widely researched in biomedical applications (Bose, Robertson, & Bandyopadhyay, 2018; Klimek & Ginalska, 2020; Wan, Wang, Ma, Sun, & Yang, 2017). Early studies have shown that using commercial hydrophobic proteins enable the hydrophobization of cellulosic cotton fibre (Opwis & Gutmann, 2011), and impregnating zein (a protein from maize) into BC can increase hydrophobicity of BC surface as well as enhancing cell attachment and proliferation when it is used as cell culture scaffold (Wan et al., 2017). Adding proteins as functional molecules to modify BC during microbial fermentation in place of traditional chemicals methods could lead to a more sustainable process, i.e. lower temperature, energy efficient and cost-effective. The modified materials are biocompatible and can be degraded in a controlled manner.

BC is, in effect, a fibrous structural component of biofilms. Biofilms in nature, however, are complex in their composition and contain a suite of functional polymers that support the survival of bacterial communities (Muhammad et al., 2020). Therefore, one method to increase hydrophobicity may found by looking at the way in which microbes already functionalise the materials they synthesise in biofilms. BslA is a hydrophobic protein that forms an elastic skin on the surface of a *Bacillus subtilis* biofilm. It possesses a highly hydrophobic cap region that is responsible for the hydrophobic nature of biofilms and it either self-assembles through conformational changes and polymerisation into a 2D lattice or is partitioned into layers within the extracellular matrix (Arnaouteli et al., 2017). Through this assembly either the hydrophilic region or hydrophobic cap of the protein will be exposed, and the selective permeability of the biofilm can, therefore, be controlled (Arnaouteli et al., 2017). The hydrophobic characteristics of BslA make it important in biofilm stability both in terms of hydration and elasticity, allowing adequate flexibility for the biofilm to respond to environmental pressures without breaking or becoming brittle (Morris et al., 2017). This strong and flexible

nature could prove useful in applications relevant to BC production, in addition to the hydrophobic advantage this protein could provide.

BslA is a structurally characterised bacterial hydrophobin and can withstand harsh liquids and chemicals, and has been shown to play a role in protection against biocides due to its non-wetting characteristics (Epstein, Pokroy, Seminara, & Aizenberg, 2011). If these characteristics can be transferred to BC, the longevity and usability of this material could be greatly improved by the addition of this protein.

Recombinant proteins may also have the advantage of interacting with the cellulose molecules directly through cellulose-binding modules (CBMs) which are short-chain proteins with a strong affinity to cellulosic materials (Linder, Salovuori, Ruohonen, & Teeri, 1996). CBMs are often found in carbohydrate active enzymes and increase the proximity of the enzyme to its substrate through their binding activity. Previous studies have utilised CBMS to enhance the binding of functional proteins with BC materials (Florea etal. 2016; Gilbert et al., 2021). By fusing these modules to C-terminus of the recombinant proteins such as sfGFP, localisation and longevity of the binding of the proteins could be extended. More broadly, through recombinant DNA techniques, CBM-fused proteins for various applications have been explored (Xu et al., 2002). For example, researchers produced bifunctional proteins consisting of synthetic polypeptide phytochelatin (EC20) fused with CBM, which were further immobilised on cellulose membrane and demonstrated to be highly effective in removing cadmium.

In our study, we hypothesize that recombinant hydrophobic protein BslA is an effective and sustainable functional molecule for modification of BC materials. This paper investigates the ability of BslA to modify BC materials. We started with an ex-situ approach to understand if the protein can influence BC properties, and then moved to an in-situ approach to explore a novel fabrication method which mimics biofilm formation. The changes in hydrophobicity as well as physical and mechanical properties were assessed. Our results demonstrate that BC can be functionally modified using recombinant proteins in a localised manner. Fabrication of functionalised BC can be achieved in a sustainable microbial fermentation process which has superiority over current chemical and physical modification.

2. Methods:

2.1 Strains, constructs and DNA assembly

DNA coding for BslA with and without CBMs and green fluorescent protein (GFP) with and without CBMs were synthesised and subcloned into pET28a by TWIST Bioscience (USA). Plasmids were transformed into *Escherichia coli* Top10 cells for verification and then transformed into *E. coli* BL21 (DE3) for expression. Constructs were verified by Sanger sequencing (DNASeq, Dundee). All sequences are provided in the supplementary materials.

The cellulose producing bacteria used in these experiments, *Komagataeibacter xylinus* 2325, was obtained from DSMZ, Germany

2.2 Culture conditions and Media

Hestrin-Schramm (HS) medium composed of 5 g L^{-1} yeast extract, 5 g L^{-1} peptone from soybean and 20 g L^{-1} glucose, 1.5 g L^{-1} citric acid, 2.7 g L^{-1} Na_2HPO_4 was used for the growth of *K. xylinus*. Cultures were initially grown with 1% (v/v) cellulase from *T. reesei* (Sigma-Aldrich, C2730) in HS medium at 30°C, 200 rpm for 3 days to reach OD_{600} between 0.9-1.2. The culture was then pelleted and washed in fresh media 4 times before being resuspended in the same volume of HS medium to remove the cellulase. Pellicles were grown by adding the starter culture to fresh HS medium at the ratio of 1: 100 and grown at 30°C static.

2.3 Recombinant Protein Expression and Purification

To express the recombinant proteins BslA-CBM, BslA, GFP and GFP-CBM, *E. coli* (DE3) transformants were grown in 10 mL of LB with 50 µg/mL kanamycin at 37°C for 16 h. Starter cultures were then used to inoculate 500 mL of fresh LB + kanamycin at ratio of 1:100 and growth until OD_{600nm} reached 1.2. Protein expression was then induced by addition of 1 mM Isopropyl-β-d-1-thiogalactopyranoside (IPTG) and the culture was incubated 37°C for 20 h. Cells were then harvested by centrifugation at 5000 x*g* for 15 min and resuspended in 40 mL buffer A (100 mM Tris, 0.5 mM NaCl and 20 mM imidazole).

Cells were lysed by sonication (QSonica ultrasonic processor) at amplitude 30% at 30 second intervals with 30 second cooling off periods for 8 cycles. This crude lysate was pelleted by centrifugation at 20000 x*g* for 20 min. The supernatant containing cell free extract (CFE) was then collected and concentration was calculated using a Bradford assay with 12 standards using NanoDrop One (Thermo Scientific™). CFE was

stored in 50% glycerol in –80°C for use in future experiments. To purify the recombinant proteins, the CFE was run down a gravity column pre-packed and pre-equilibrated with TALON metal affinity resin (CloneTech). The resin was washed twice with buffer A before elution of protein in 20 mL buffer B (100 mM Tris, 0.5 mM NaCl, 500 mM imidazole). Concentration of purified protein was calculated as previously described. Protein aliquots were then stored either in glycerol as previously described or freezer-dried prior to storage at - 80°C. Expression was confirmed by visualisation on Coomassie blue stained SDS-PAGE gel.

2.4 CBM binding property testing

The surface binding property of recombinant proteins with CBM were evaluated using recombinant GFP proteins. *K. xylinus* culture was grown in 12 well plates statically for 10 days, the BC pellicle washed in distilled H_2O twice and air dried at 20°C (+/-2°C) prior to being submerged in 10 mL of 5 mg/mL GFP or GFP-CBM CFE for 4 h. As a material control, 1 cm x 2 cm parafilm was also submerged in the same amount of GFP-CBM CFE. Post-coating, samples were then washed briefly in dH_2O twice and air dried at 20°C (+/-2°C) on glass slides. The photographs were taken under UV to observe the amount of GFP on the pellicles.

2.5 Design of Experiments for optimisation of BC preparation and protein application

Design of experiment (DOE) for the optimisation of BC preparation and protein application to maximise hydrophobicity was performed using JMP pro software version 16 (SAS institute, North Carolina, USA). BC pellicles were prepared by growing as previously described in 2.2. After pellicles were collected and media was removed by washing in dH_2O, several variables were identified and tested: namely, washing in 100% (v/v) IMS for 12 h, washing in NaOH at 90°C for 60 min, dehydration through lysophilisation, and dehydration through air-drying at 20°C (+/-2°C). Recombinant protein was then applied to the dry pellicle surface as either CFE or purified protein, both at a concentration of 10 mg/mL. BslA with and without CBMs were tested and following the drip coating the pellicles were either dried at 20°C (+/-2°C) or at 50°C (+/-5°C). In total 6 factors were defined, and a definitive screening design was chosen. The JMP software was used to generate 18 experiments with the design providing an estimate on all main effects, as well as a limited number of first order interactions. Hydrophobicity was measured by pipetting a 10 µL droplet of sterile H_2O onto the surface and determining the length of time the droplet held its shape before diffusing across the surface (0-4 /10 seconds). This experiment was carried out in triplicate and empty vector controls were included.

2.6 Ex-situ BC modification

The ability of BslA to infer a hydrophobic coating was initially tested on filter paper. For the test, 500 µL of 10 mg/mL BslA-CBM CFE was pipetted either in the centre or in a ring around the outside of circular filter paper (90 mm diameter), and then 1 ml of water coloured with commercial food dye (Wilton) was used to flood the filter paper. The experiments were recorded as photographs and video.

Similarly, the changes of BC surface hydrophobicity using BslA and BslA-CBM was assessed by adding 1 ml of 500 µg/mL, 250 µg/mL, 50 µg/mL CFE onto pellicles prepared, as described in 2.2. The same volume of equal concentration CFEs from *E. coli* transformant with empty vector and buffer A were used as controls.

2.7 In-situ modification

A starter culture of *K. xylinus* was prepared and inoculated into a 24 well plate as described in 2.2. CFE of BslA, and BslA-CBM were sterilised using 0.2 µm filter and added to wells in one of the following concentrations: 50 µg/mL, 250 µg/mL, or 500 µg/mL. *K. xylinus* culture with no CFE added was used as a negative control. To prevent pellicle formation, all cultures contained 1% cellulase. The plate was incubated at 30˚C with medium shaking settings for 96 h inside a Synergy HT plate reader (BioTek), and the absorptions at 600 nm were recorded at every hour. All experiments were performed in triplicate.

A starter culture of *K. xylinus* was prepared and inoculated into a 24 well plate as described in 2.2. CFE of BslA, and BslA-CBM were sterilised using 0.2 µm filter and added to wells in one of the following concentrations: 500 µg/mL, 250 µg/mL, or 50 µg/mL. *K. xylinus* culture with no CFE added and 500 µg/mL empty vector CFE were used as negative control and positive control respectively. The plate was incubated at 30°C and pellicle formation was monitored for 5 to 10 days before pellicles were collected and treated for WCA analysis. All experiments are performed in triplicate.

To determine if BslA had been incorporated into the pellicle, the supernatant was collected and where present, pellicles were collected and washed in PBS for 10 minutes twice. Pellicles were then degraded by shaking in 10 mL PBS with 2% (v/v) cellulase from *T. reesei* for 24 h. The digested pellicles were run on SDS-PAGE.

To investigate the impact of BslA on BC film mechanical properties, larger experiments were set up in 2 L conical flasks in which *K. xylinus* was grown in 200 mL HS media with 250 µg/mL BslA-CBM CFE. After

10 days fermentation, pellicles were harvested, washed briefly in dH_2O twice and then air dried at 20℃ (± 2℃).

2.8 Water contact angle tests

Water contact angle (WCA) tests were carried out to measure hydrophobicity on three areas of each sample, i.e., 2 µL droplets were deposited on flat BC surfaces using a droplet shape analyser (DSA) with built-in camera (Krüss) to record the droplet. ImageJ was used to measure the angle of water droplet at the point of contact.

2.9 Microscopic analysis

Atomic Force Microscopy (AFM) was used to image the BC film post drying, visually observe the protein coating, and measure surface roughness. Samples were prepared by drying at 20℃ (+/- 2℃) on glass slides. Topographic images of the cellulose coated with BslA and BslA-CBM were acquired using a commercial AFM system (Veeco DI3100, Bruker Corporation). Intermittent contact mode imaging techniques were applied using a cantilever with a stiffness of 26 N/m and tip radius of < 7 nm (OTESPA, Apex Probes) by oscillating the cantilever over the samples. Scanning electron microscopy (SEM) was also used to investigate the morphology and macrostructure of the BC film. Samples were prepared for SEM analysis by coating with palladium and then imaging using a TESCAN Mira 3 in conjunction with Alicana 3D imaging software.

2.10 Mechanical property tests for in-situ modification

Three strips were cut from each BC film according to measurements dictated by ASTM D883, giving a total of 9 samples per treatment. The mass and volume of each strip was then recorded, and the tensile strength of this material was measured according to the standard protocol using Instron (Instron 68TM-50 with Bluehill Universal Version 4.21 software). The Young's modulus was calculated using stress-strain curves and plotted in reference to the density of each sample.

2.11 Statistical analyses

Statistical analysis of results obtained from contact angle measurements and materials testing was carried out by running Analysis of Variance (ANOVA) in R (RStudio Team, 2020). All experiments were performed in biological and technical triplicates (n=9). P-values were obtained following normality checks, and post-hoc Turkey tests were carried out, P-values < 0.05 were determined to be significant.

3. Results:

3.1 Expression of recombinant proteins and CBM binding tests

Recombinant BslA, BslA-CBM, GFP and GFP-CBM, were successfully overexpressed *E. coli* and the yields of recombinant proteins were compared on 12% SDS-PAGE (figure S1). Comparing BC treated with GFP CFE, BC treated GFP-CBM CFE and parafilm treated with GFP-CBM CFE shows that the CBM significantly increased the binding of GFP proteins on the BC pellicle (figure 1).

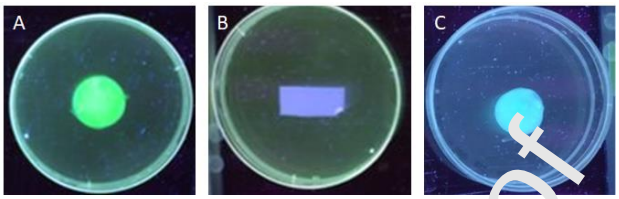


Figure 1. CBM binding tests*. A. BC pellicles shaken in 10 mL of 5 mg/mL GFP-CBM CFE; B. Parafilm shaken in 10 mL of 5 mg/mL GFP-CBM CFE; and C. BC pellicles shaken in 10 mL of 5 mg/mL GFP CFE.*

3.2 DOE Optimised BC modification protocol

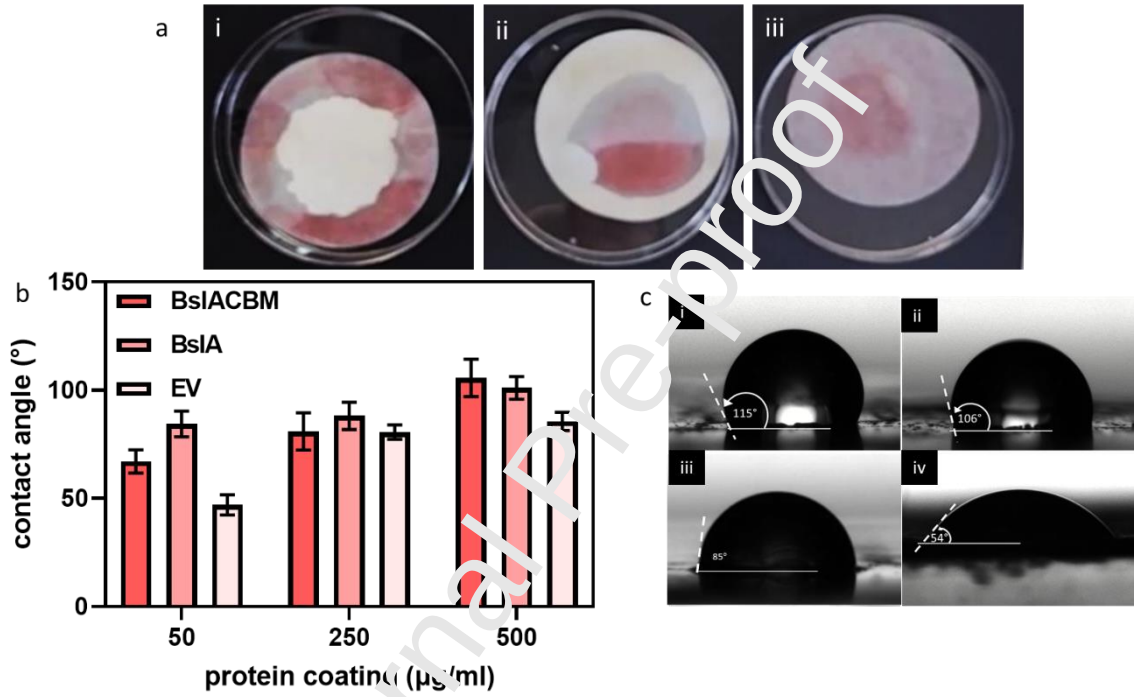
A DOE definitive screening design approach was used to assess the optimal protocol for pre-treatment and coating of a BC surface. Identification of 6 factors was used to define 18 experiments that tested which factors resulted in the most hydrophobic BC surface. It was determined that pre-treatment of the BC prior to protein coating (washing with solvents or NaOH) showed no significant difference in hydrophobicity. There was also no significant difference in the hydrophobicity of the BC between the different drying methods (lyophilised vs air dried at any temperature). The most significant increase in BC surface hydrophobicity was seen when using protein CFE instead of a purified protein sample and therefore all future experiments were carried out using CFE on airdried pellicles with no prior wash steps (figure S2).

3.3 Improvement of BC surface hydrophobicity through ex-situ coating

The impact of BslA as a hydrophobic coating molecule was tested on filter paper treated with BslA-CBM CFE before being flooded with coloured water. We show that the water was not able to enter to the areas treated with BslA (figure 2 (a) and S video1), confirming the hydrophobic nature of this protein.

Air-dried BC pellicles were treated with a range of concentrations of BslA and BslA-CBM CFEs and the surfaces were analysed through WCA tests. The increase in WCA was observed from all the BC treated samples (figure 2 (b) & (c)). When BC was coated with 250 µg/mL of BslA and BslA-CBM CFEs, the WCA

of both materials increased with the WCA at 88°± 6.28 and 81°± 8.58, respectively and hydrophobicity (WCA > 90°) was achieved when the BC pellicles were coated with 500 µg/mL CFEs. Further analysis has shown that coating at a higher concentration did cause a significant increase to the hydrophobicity when a CBM was used. The contact angle of the 500 µg/mL concentration CFE coating was significantly higher at 105°± 8.62 compared to that of 50 µg/mL concentration CFE coating (67.055°± 5.462°) with the P value of 0.048. Although the WCA for BC coated with 500 µg/mL of BslA without CBM also increase compared to 250 µg/mL, the change was not statistically significant. Although unexpected, it is likely that the protein is



washed off to a low concentration uniformly, despite the difference in the initial concentration. The hydrophobicity of the BslA treated samples was significantly higher compared to the positive control at a high and low concentration ($P < 0.05$).

Figure 2. Hydrohpobicity tests. *(a)Testing hydrophobicity of BslA on filter paper. (i) BslA-CBM CFE wasapplied in the centre; (ii) BslA-CBM CFE was applied on the outer edge; (iii) No CFE applied. (b) Comparison of WCA for samples treated with CFE of BslA, BslA-CBM and empty vectors (positive control). The concentration of the CFEs are 50 µg/mL, 250 µg/mL and 500 µg/mL respectively. (c) Water contact angel images for the BC treated with (i) 500 µg/mL BslA-CBM CFE; (ii) 500 µg/mL BslA CFE; (iii) 500 µg/mL empty vector CFE; and (D) Buffer A only.*

3.4 Characterization of hydrophobic BC though ex-situ coating

The AFM images were comparatively analysed and revealed that the surface of the BC, when coated with BslA-CBM, was completely covered with proteins at a high concentration and the fibres became more visible with decreasing concentration (figure 3). No significant morphological change was observed in terms

of fibre thickness, length and aspect ratio. Within the 3D structured protein layer, we observed the recombinant proteins as aggregates at all concentrations of CFE coating when applied through surface dripping, although it changed from large spheres at low concentration (50 µg/mL) of CFE to protein particle films at high concentration (500 µg/mL) of CFE. It is also notable that the coverage with BslA-CBM CFE coating (figure 3 (a)-(c)) is significantly higher than those without CBM (figure 3 (d)-(f)). In addition, a surface roughness test was conducted, as it has been reported that hydrophobic properties of surfaces were enhanced by increasing surface roughness (Wan et al., 2017). The surface roughness of CFE coated BC was quantified by analysing AFM images (5 x 5 µm scans) and the results revealed that the BC surface treated with empty vector CFE had the lowest roughness. Roughness of BC increased with 50 µg/mL CFE of BslA and BslA-CBM as protein aggregates formed on the surface (figure 4). However, the roughness values decreased as the protein concentration increased, despite the surface hydrophobicity continuously increasing as shown in figure 2.

The presence of the BC fibres can be observed in the line scans extracted from the AFM images (figure 3(h)-(i)); the higher amplitude and lower frequency oscillations present show the underlying BC fibres with lower amplitude and higher frequency oscillations present show the BSLA coating. In the line scan extracted from the sample with the BSLA-CBM coating (figure 3(i)), the higher frequency oscillations have a smaller amplitude, implying better adhesion to the BC fibres when compared to the sample without CBM (figure 3(h)).

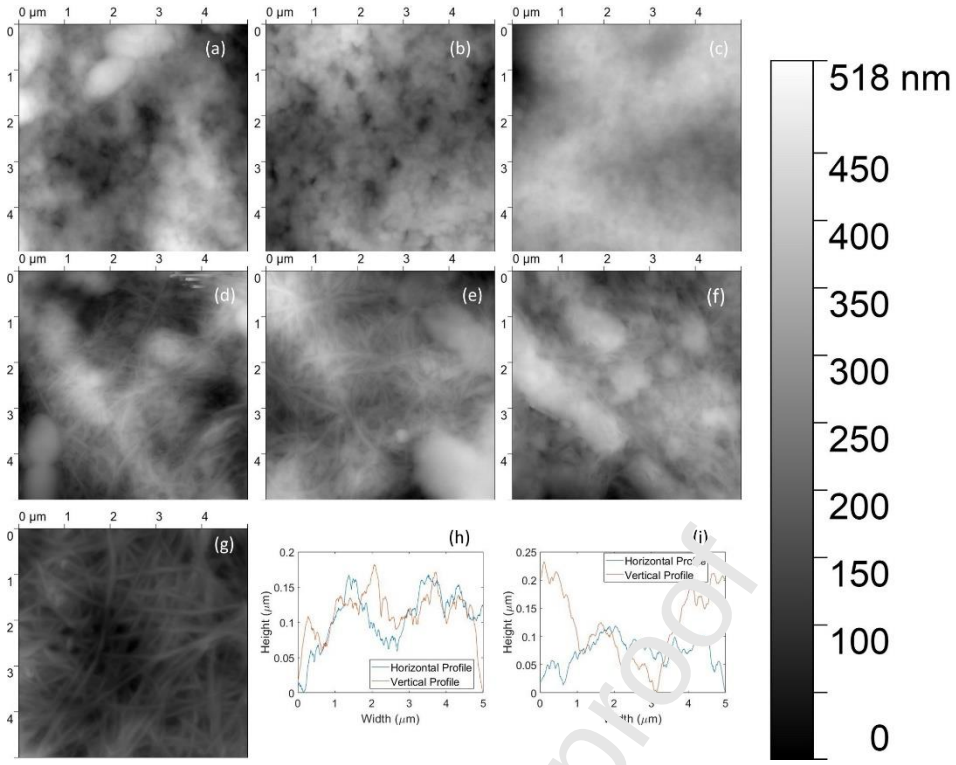


Figure 3. Atomic force microscopic images to show the surface morphology of ex-situ modified BC pellicle. *(a)-(c) BC pellicles were treated with 50 µg/ml, 250 µg/ml, and 500 µg/mL BslA-CBM CFE; (d)-(f) BC pellicles were treated with 50 µg/mL, 250 µg/mL, and 500 µg/mL BslA CFE; and (g) BC pellicle was treated with 500 µg/mL empty vector CFE. (h) Line Profile extracted from (e) and (i) Line profile extracted from (b).*

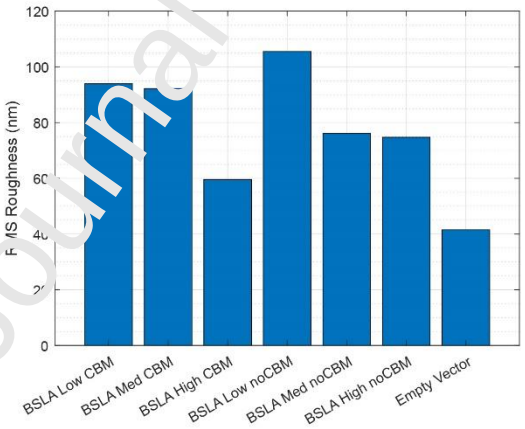


Figure 4. Surface roughness values for ex-situ modified BC pellicle. *Area 5 x 5 µm. 50 µg/mL, 250 µg/mL, and 500 µg/mL of CFE are presented as low, medium and high respectively in the figure.*

3.5 BslA in-situ modification of BC pellicle

Initially we investigated the impact of recombinant CFEs on *K. xylinus* growth and BC formation. The growth curves of *K. xylinus* culture with 50 µg/mL, 250 µg/mL, or 500 µg/mL CFE of BslA or CFE of BslA-CBM and control culture (no CFE was added) were plotted (figure S3). While the growth in the CFE-treated

K. xylinus culture was detected slightly earlier compared with the control culture, the overall growth pattern was the same, indicating that CFE treatment has no negative effects on the growth of *K. xylinus*.

To modify the BC property in-situ, 50 µg/mL, 250 µg/mL and 500 µg/mL CFEs were added at beginning of fermentation. The addition of CFE of empty vector and low concentration of BslA and BslA-CBM (50 µg/mL) had no impact on the BC formation, and the cellulose formed under the surface of the liquid / air interface can be observed from day 2. However, upon addition of 250 and 500 µg/mL of CFE containing recombinant proteins, surface pellicle formation was notably altered (figure 5). The addition of 250 µg/mL of CFE of BslA produced surface pellicles on average after 5 days growth, and fermentation with 500 µg/mL BslA-CBM was not able to produce BC pellicle before 10 days at this scale.

Figure 5. Impact of recombinant proteins CFE on BC pellicle formation with 5 days incubations*. (a) 1A-1C. cultures with 500 µg/mL BslA-CBM CFE; 2A-2C. cultures with 250 µg/mL BslA-CBM CFE; 3A-3C. cultures with 50 µg/mL BslA-CBM CFE; 4A-4C. cultures with 500 µg/mL BslA CFE; 5A-5C. cultures with 500 µg/mL BslA CFE cultures with 500 µg/mL BslA CFE; 6A-6C. cultures with 50 µg/mL BslA CFE; D1-D3. cultures with 500 µg/mL empty vector CFE; D4-D6. Culture with no CFE. (b) A Heat map representing the growth data from the plate experiment.*

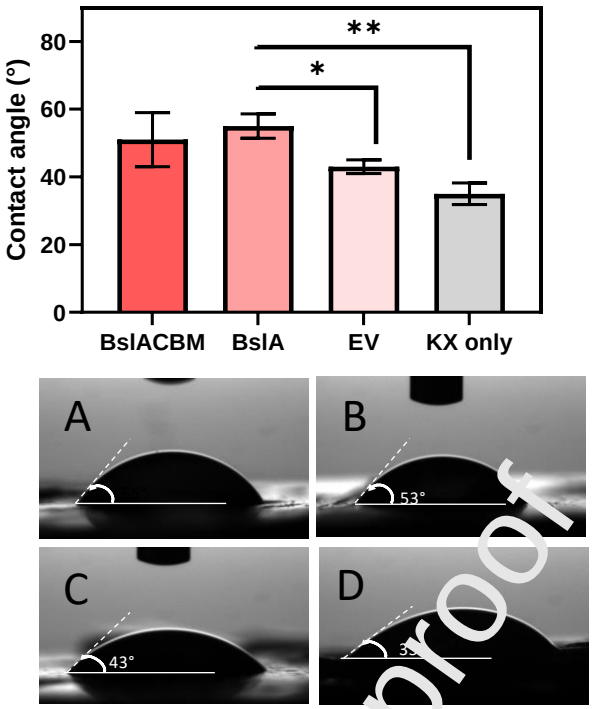


Figure 6. Water contact angle tests for CFEs in-situ modified BC pellicle. *Comparison of WCA for samples grown with 250 µg/mL CFE of BslA (A), BslA-CBM(F) and empty vectors (C) and no CFE (D).*

The WCA tests for the in-situ BC modification with various CFEs (after 10 days growth) show that, although the BCs treated with recombinant proteins have increased surface hydrophobicity (i.e. WCA increased from 35°± 2.67° to 55°± 4.39°) the materials still retained their hydrophilic nature (figure 6). There was a significantly higher WCA in samples formed with BslA compared to the empty vector (P = 0.0467) and *K. xylinus* only control (P = 0.0092). However, there was no significant change in WCA for those BC films grown with BslA-CBM other than when compared to *K. xylinus* only (P = 0.0254).

Half of the BC pellicles modified with BslA-CBM were digested using cellulase and the degradation products were harvested and visualised through SDS-PAGE gel. The gel image (figure S4) shows that there is a clear band in the lane of recovered BC through in-situ treatment with BslA-CBM CFE. There is a dominant band which is over 25 kDa and in line with the overexpressed BslA-CBM when CFE was visualised on the gel. This result indicates that the recombinant BslA-CBM proteins remained undegraded during the in-situ modification process.

In contrast to the ex-situ coating experiments reported above, the SEM images (figure 7) of in-situ modification revealed the BC surface was most densely covered by proteins in the BslA CFE treatment but

not BslA-CBM CFE treatment. Although protein clusters can be identified on the surface of BslA-CBM treated BC (figure 7 (a)), in the case of BslA, the surface was fully covered by the recombinant proteins (figure 9 (b)). AFM images (figure S5) show that in-situ treatment with BslA-CBM led to a 3D distribution of proteins, i.e. some protein aggregates can be observed under the cellulose fibre network. This may lead to slightly loose interaction between fibres within the BC cellulose 3D structure. In contrast, there was no 3D incorporation observed following in-situ treatment with BslA without CBM and the protein aggregates formed a 2D layer on the top BC surface. In addition, there was little difference in surface morphology of BC film form with BslA-CBM CFE and empty vector CFE (figure 7). Again, there was no obvious difference in the cellulose fibres in terms of length, diameter and aspect ratio in all the samples from in-situ modification (figure S5 and figure 7).

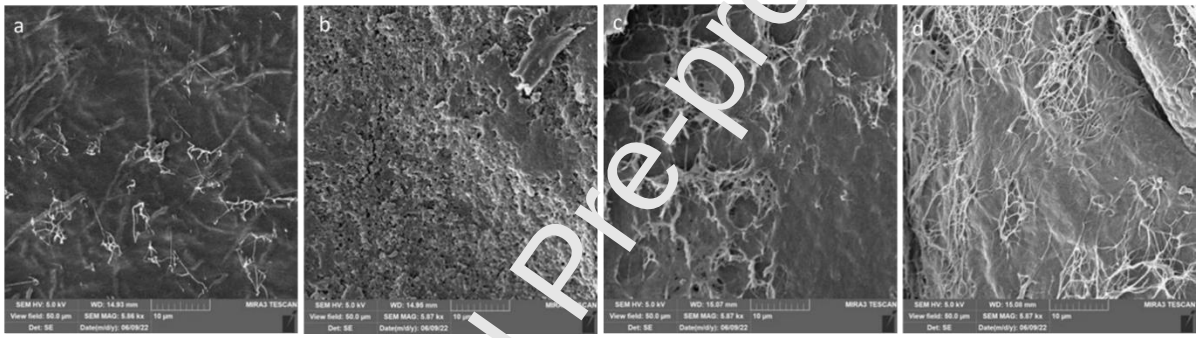


Figure 7. Scanning electron microscope (SEM) images of BC surface. *BC in-situ modified by (a) 250 µg/mL BslA-CBM CFE; (b) 250 µg/mL BslA CFE; (c) 250 µg/mL empty vector CFE; and (d) no CFE. Red arrows on (a) indicate proteins on the surface, it should be noted that proteins cover the entire surface on (b).*

3.6 The mechanical properties of BC with BslA in-situ modification

Larger scale experiments carried out to investigate changes to BC mechanical properties through in-situ modification yielded significant results. Samples grown with BslA with or without CBMs took considerably longer to dry out than previously observed. BC treated with BslA-CBM had a significantly higher tensile strength than those with the empty vector CFE (P = 0.0357) (figure 8 (a)). Likewise, those samples treated with recombinant proteins had a significantly higher percentage of elongation during Instron testing compared to empty vector with p values of 0.0182 and 0.0255, respectively (figure 8 (b)). Calculation of the BCs elasticity revealed those with BslA and without CBMs had significantly lower Young's modulus than empty vector control samples, with p-values of 0.0091 and 0.0149 (figure 8 (c)). By plotting the Young's modulus against density (figure 8 (d)), comparisons to other materials can be made, specifically by comparing to a chart of standard materials (CES EduPack 2019, ANSYS Granta © 2020 Granta Design), it can be concluded that this treated BC is most closely related to elastomers.

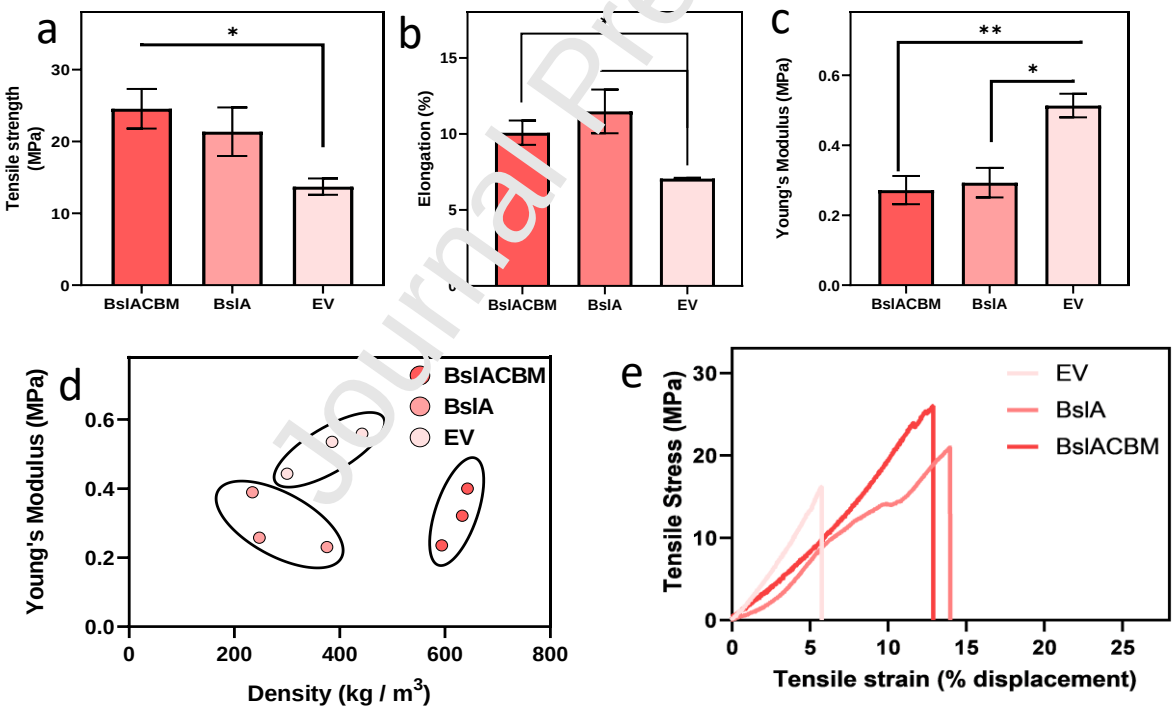


Figure 8. Materials testing of BC in-situ modified with 250 µg/mL CFEs of BslA-CBM, BslA and empty vector (EV). *(a) Tensile strength testing; (b) Elongation testing; (c) Yong's modulus testing; and (d) A diagram of Yong's Modulus vs Densit (e) Representative stress strain curve from tensile strength testing*

Discussion

In this study, the ability of recombinant protein, BslA, to act as a functional molecule to modify BC was investigated. Ex-situ modification of BC with CFE of BslA with and without CBM significantly increased the BC surface hydrophobicity and this increase correlated with increasing concentration of CFEs. When BC was coated with BslA-CBM, the average WCA reached 105°± 8.62°, and the highest WCA observed was 120°. This result is comparable with many existing BC hydrophobicity treatments which use physical and chemical approaches. For example, BC surfaces modified through the 'click' polymerization approach leads to the surface WCA around 102° (Krishnamurthy, Lobo, & Samanta, 2020) and BC nonwovens functionalised by poly fluorophenol increase the WCA from 54° to 120° (Song, Silva, Cavaco-Paulo, & Kim, 2019). In this study, the WCA of untreated BC was 51° on average, which is slightly higher than that reported in other studies, for example, 30°C to 33°C (Ybañez & Camacho, 2021). We suspect the variation was caused by different post-production processes, and the status of BC during analysis, i.e. here, air-dried BC was tested and Ybanez and Camacho tested wet BC

The results of AFM analysis regarding surface morphology of the samples agree with other studies which used hydrophobic proteins to modify material surfaces. Winandy et al. (Winandy, Hilpert, Schlebusch, & Fischer, 2018) observed large aggregates of fungal hydrophobins on a glass surface, and Wan et al (Wan et al., 2017) found the zein protein formed large spheres at lower concentration on BC and protein particle films at higher concentration. However, following surface roughness analysis of CFE treated BCs in conjunction with surface hydrophobicity, we found that our results are different from the observations of zein coated BC. In that study, the roughness value for treated BC increased from 1 mg/mL zein protein to 5 mg/mL zein protein but decreased from 5 mg/mL to 20 mg/mL and the hydrophobicity of treated BC pellicles had a similar trend (Wan et al., 2017). It is difficult to make a direct comparison between these two studies, as i) Wan et al reported higher concentration of proteins (1-20 mg/mL vs 50-500 µg/mL CFEs); and ii) different coating methods were applied (submersion compared to dripping). We suspect that after 1 ml of 500 µg/mL CFE was dripped on the BC, the proteins had saturated the local surface creating an even layer of proteins as opposed to generating a patchy, uneven surface as observed at low concentrations. This result is particularly true in the case of BslA-CBM where the coating is significantly higher than on BC coated with BslA without CBMs (figure 4 (a)-(c) vs (d)-(f)) and the BC coated with empty vector CFE has the smoothest

surface. The hydrophobic properties provided by a high concentration CFE are less determined by the roughness of the surface, but rather to the hydrophobic nature of the BslA.

While some applications could benefit from a rougher surface such as adhesion in tissue engineering (Wang et al., 2019), other applications necessitate a smooth surface such as in paper fabrication (Fillat et al., 2018) and as a food additive (Choi, Rao, Zo, Shin, & Han, 2022). Finding a balance between hydrophobicity and surface roughness has, so far, been a challenge, but results presented here demonstrate that BC treated with 500 µg/mL BslA-CBM generates a surface which is significantly more hydrophobic than the untreated control, whilst not being significantly rougher.

An interesting observation was that the hydrophobicity of the BslA-CBM samples is lower than that of the BslA without CBM in some instances, particularly at lower concentrations, possibly due to the orientation of cellulose binding to BslA-CBM. In nature, CBM is a part of cellulase modular structure and it connects with a core catalytic domain via a flexible glycosylated linker (Quinlan, Teter, & Xu, 2010). CBM binds the hydrophobic patches of the cellulose through the aromatic amino acids via van der Waals interactions (Griffo et al., 2019), thus enhancing cellulose hydrolysis by facilitating enzyme synergy via proximity-effects (Pérez & Tvaroška, 2014; Quinlan et al., 2010). In this study a double CBM was attached to the C-terminus of the BslA, which has been shown to significantly increase binding to cellulosic surfaces compared to a single CBM (Levy, Nussinovitch, Shpigel, & Shoseyov, 2002). However, CBMs are themselves planar hydrophobic surfaces, although less hydrophobic than the cap of BslA (figure 9). Ideally, the hydrophobic cap region on BslA would be oriented outward to infer hydrophobicity, however the hydrophobic residues on the cap may face inwards, due to its possible binding with cellulose surface through van der Waals interactions. In addition, a hydrophilic region exists on the N-terminus of our BslA sequence (residues 27 – 43) (Bromley et al., 2015), consequently, when the hydrophobic cap bound to the BC surface the hydrophilic tail and the CBM are oriented away from the BC surface. Therefore, despite the likelihood of more protein remaining bound to the BC, due to the exposure of hydrophilic regions, the hydrophobic function of this protein may not be fully exploited. In this instance, the observed lower WCA can be attributed to the CBMs. To counteract this, CBMs could be designed to be more highly specific to BC, or the hydrophobic region of BslA could be manipulated to have a lower affinity to BC. Additionally, the hydrophilic region would be removed from BslA. These alterations could guarantee the exposure of hydrophobic region when

bound to the BC surface (Figure 10), resulting in a substantial increase in surface hydrophobicity. This change may also offer a way of programming the material by altering the mix of CBM locations to provide greater or lesser degrees of hydrophobicity.

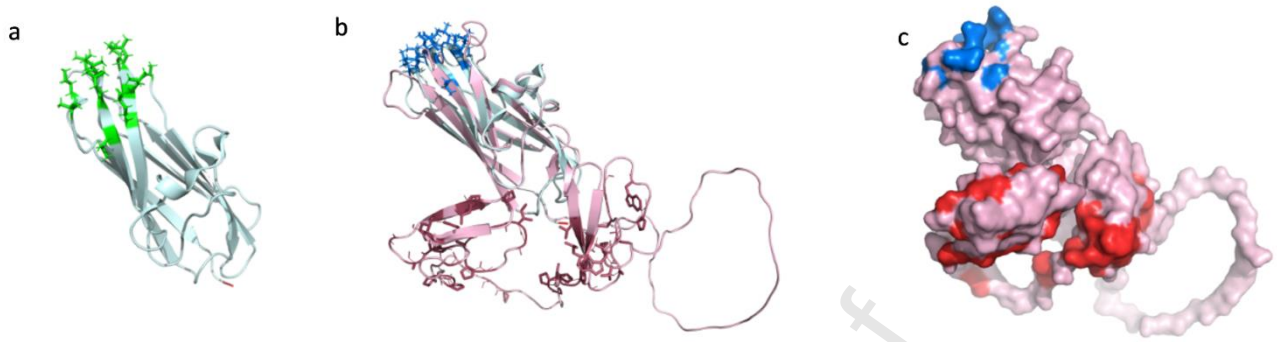


Figure 9: BslA structures *(a) The crystal structure of a BslA monomer. The hydrophobic cap region (green sticks) is located near the N-terminus whereas the CBM is located at the C-terminus (Red). (b) A comparison of the predicted structures of BslA-CBM (BslA, Cyan - PDB 2NB4U; Pink - as predicted by AlphaFold2 (Mirdita et al., 2022) the structure of hydrophilic tail region has been removed due to poor prediction). The hydrophobic cap region (blue) is located near the N-terminus with the dCBM (red) located near the C-terminus. Hydrophobic residues are highlighted. (c) A surface view of the predicted structure of BslA-CBM.*

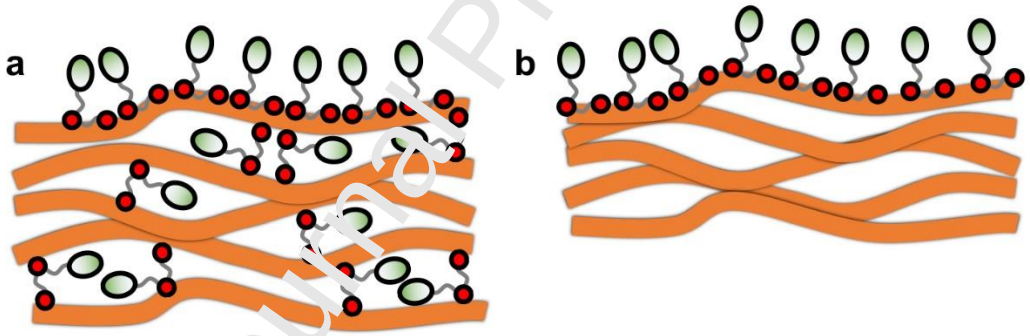


Figure 10: Ideal interaction of BslA-CBMs with BC fibres *(a) in-situ modification and (b) ex-situ modification*

A final observation is that the WCA for the BC treated with CFE containing an empty vector did change particularly at higher concentrations (250 µg/mL and 500 µg/mL). Although these results are significantly lower than the BC treated with BslA CFEs. We believe there is likely to be some lipids and proteins present in CFE which provide a weak hydrophobic coating for the BC surface. Nevertheless, this observation does not discredit the ability of recombinant BslA produced by *E. coli* to act as a hydrophobic coating.

Results of the in-situ modification experiments showed higher concentrations of BslA and BslA-CBM CFE delay the formation of the BC pellicle. We suspect that due to the amphiphilic nature of BslA, the protein molecules naturally sit at the liquid and air interface, thus occupying the area where pellicle formation would

normally occur. Eventually, the pellicle is formed and the WCA did increase when BslA was present, however, at 250 µg/mL it remained hydrophilic (WCA < 90°). There were two possible explanations for this observation: i) the BslA-CBM was not incorporated into the BC structure during the pellicle formation or ii) BslA-CBM is incorporated into the middle layers of pellicle rather than sitting as a coating on the topmost surface. To investigate further, the CFE in-situ modified BC pellicles were analysed through both cellulose degradation and microscopic images. From the SDS-PAGE, we can conclude that there is a significant concentration of protein incorporated in the treated BC BslA-CBM composite. In conjunction with the observation from AFM and SEM we suspect that BslA-CBM has been incorporated into the middle layers of growing pellicle whereas BslA remained on the BC surface. In addition, the lack of surface morphology difference may also explain the similarity in surface hydrophobicity between samples grown with CFE with empty vector and those grown with BslA-CBM.

Mechanical tests with large scale in-situ modification with CFEs suggested that treatment with BslA and BslA-CBM significantly changed BC properties in terms of tensile strength, percentage elongation and Young's modulus. Compared to other reports, the decrease observed in Young's modulus, on addition of proteins, is similar to that reported by Szymańska-Chargot et al. (Szymańska-Chargot et al., 2019) where the addition of chitosan acts as a plasticiser of the cellulose. The Young's modulus values obtained in our study are lower than those reported elsewhere (Hsieh, Yano, Nogi, & Eichhorn, 2008) predicted a Young's modulus of 114 GPa), and this may be because our samples are considerably thinner (0.3 mm on average) due to the delay of the BC formation during the 10-day growth period. There are, however, similar tensile strength values reported for dried BC. For example, cellulose treated with gelatin had a tensile strength of < 4 MPa (Haque, Kurokawa, & Gong, 2012), or that treated with chitosan had a maximum tensile strength of 30 MPa (Liang, Wang, & Chen, 2019). Likewise, the tensile strength of BC prepared via the kombucha method yielded a tensile strength of 10-16 MPa (Molina-Romero, Arteaga-Ballesteros, Guevara-Morales, San Martín-Martínez, & Vieyra, 2021). One explanation for this is the interruption of hydrogen bonding between cellulose fibres (–OH) due to the presence of the CFE (–OH and $–NH_2$). Therefore, bonds forming between the BC and CFE alter the network of the matrix and promote a less brittle and stronger material. Again, by manipulating the sequences of recombinant proteins, potentially we can create an ideal interaction of BslA-CBMs and cellulose fibres (Figure 10a).

Materials often need to be strong and flexible to have sufficient durability and longevity. To create a natural material that meets these demands has been an industry-wide challenge, and like creating hydrophobic surfaces, current methods rely heavily on chemical treatments (Martin, 2012; Wu et al., 2017). Further exploration of these properties in BslA-CBM treated BC to investigate the potential changes to gas permeability or fire retardancy could reveal BslA-CBM treated materials to have additional functional properties. In addition, Morris and his colleagues characterised three orthologues of BslA and they are from *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus pumilus* respectively. All three proteins share a high degree of similarity to *B. subtilis* BslA (used in this study), while still possessing variations which lead to distinctive physical characteristics. In particular, BslA from *B. licheniformis* has been shown to be more hydrophobic than *B. subtilis* BslA and other orthologues – in this orthologue, the polar serines are replaced by nonpolar amino acids (A76 and G152) in the hydrophobic cap region (Morris et al., 2017). This study demonstrated that the source of biofilm hydrophobins (BslA) can be extended to many other species, and through an engineering biology approach the performance of modified BC materials can be improved.

Overall, this work represents a promising strategy for the use of recombinant proteins for functional BC material fabrication. Different modification approaches led to different BC properties and the versatile methodology can also applied to other proteins or peptides. Our work also provides some initial steps to develop a fabrication strategy for BC which is inspired by biofilm formation by adding functional proteins into BC, creating a more useful material. BC is a sustainable source material that offers significant opportunities for applications beyond industrial biotechnology. The use of a biological approach to functionalise cellulose, in our case adding hydrophobicity, may also be a more sustainable approach than energy intensive physical treatments or potentially polluting chemical treatments. To this end recombinant proteins could be ideal functional molecules to achieve this purpose as

1) The functional molecules can be optimised through manipulation of the sequence using molecular biology approaches
2) The production of the molecules can be achieved though sustainable microbial fermentation processes
3) The functional BC materials produced using these molecules are biocompatible and biodegradable

With development in the field of Engineering Living Materials (Gilbert & Ellis, 2019; Gilbert et al., 2021; Nguyen, 2017), this approach could also be pushed further to fabricate functional BC materials using

microbial cell factories consisting of BC producing bacteria and engineered microbial cells which could produce recombinant protein extracellularly. With the right controls, such a multi-microbial approach could lead to spatially organised functions as seen in real biofilms, allowing tuning of the function of BC to create complex composite and functionally graded materials.

5. Conclusions

This paper demonstrated the ability of recombinant BslA, as a functional molecule, to modify BC properties. Ex-situ modification of BC with CFE of recombinant BslA has been shown to increase hydrophobicity. This increase is comparable to chemical and physical treatments, if not better. The localisation of protein binding was strengthened through the addition of CBM. We believe that hydrophobicity can be further improved by rational sequence design. Results from in-situ modification show that BslA can cause structural and mechanical changes to BC fibre network to create a stronger, less brittle material which could increase its potential in a wide range of applications. The methods described outline how these functional properties can be embedded within a growing BC pellicle, with potential for localisation, without the need for chemical coating or high energy inputs. Overall, this study is the proof of concept that recombinant proteins can be used in the fabrication of functional BC materials.

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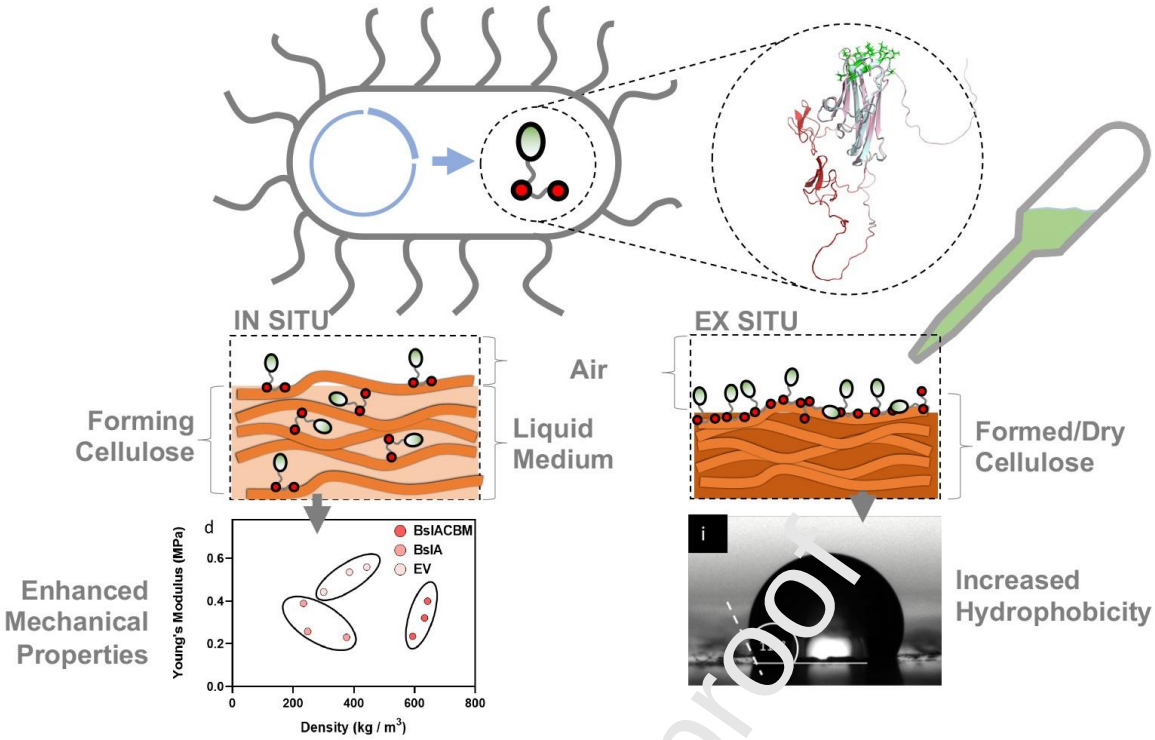
Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Graphical abstract