Assessing effectiveness of Komagataeibacter strains for producing surface-microstructured cellulose via guided assembly-based biolithography

Marcello Brugnoli1, Francesco Robotti2, Salvatore La China3, Kavitha Anguluri1, Hossein Haghighi1, Simone Bottan2, Aldo Ferrari2,* & Maria Gullo1,*

In this study, a medical device made of surface microstructured bacterial cellulose was produced using cellulose-producing acetic acid bacteria wild-type strains in combination with guided assembly-based biolithography. The medical device aims at interfering with the cell’s focal adhesion establishment and maturation around implantable devices placed in soft tissues by the symmetrical array on its surface. A total of 25 Komagataeibacter strains was evaluated over a three-step selection. In the first step, the ability of strains to produce a suitable bacterial cellulose layer with high production yield was examined, then nine strains, with a uniform and smooth layer of bacterial cellulose, were cultured in a custom-made silicone bioreactor and finally the characteristics of the symmetrical array of topographic features on the surface were analysed. Selected strains showed high inter and intra species variability in bacterial cellulose production. The devices obtained by K2G30, K1G4, DSM 46590 (Komagataeibacter xylinus), K2A8 (Komagataeibacter sp.) and DSM 15973T (Komagataeibacter sucrofermentas) strains were pouch-forming with hexagonal surface pattern required for reducing the formation of fibrotic tissue around devices, once they are implanted in soft tissues. Our findings revealed the effectiveness of the selected Komagataeibacter wild-type strains in producing surface microstructured bacterial cellulose pouches for making biomedical devices.

The search for biopolymers with innovative attributes is a challenge for the biotechnological industry. In this frame, bacterial cellulose (BC) in the native and functionalized form has received extensive attention due to features, such as high-water holding capacity, high light transparency, non-toxicity, purity and biocompatibility. Based on the structural and safety characteristics of BC and its derivates, this biopolymer has been proposed in several fields such as food, textile, pharmaceutical, biomedical, cosmetic, environmental and engineering applications. According to the United States Food and Drug Administration (FDA), BC is is “generally recognized as safe” (GRAS). Recently, the European Food Safety Authority (EFSA) Scientific Panel on Biological Hazards (BIOHAZ) included the species Komagataeibacter sucrofermentans in the list of QPS-recommended biological agents, intentionally added to food. Previous studies highlighted the absence of BC cytotoxicity on mouse fibroblast cells and its suitability as the carrier of active medical and cosmetic formulations. For instance, BC could be used as a vehicle for antibiotics or medicines, allowing their transfer to the wound. At the same time, it acts as a physical barrier against external infections. Moreover, BC is adaptable to the wound surface and provides an exudate absorption thus it is possible to use it as a matrix for the epithelialization of burns even of third-degree. BC grafts might potentially reduce the rejection rates of transplanted corneas and improve the treatment of eye diseases (e.g., age-related macular degeneration) mainly due to the augmenting local neovascularization, diminishing side effects, and surgical recovery intervals. However, it is widely known that the human body recognizes the foreign material immediately after implantation, and it could trigger an
of the topographical features, which physically interfere with the establishment and maturation of focal adhesion\(^21,24\). Biological recognition\(^22\) avoiding the formation of fibrotic tissue around implantable devices placed in soft tissues.

AAB strains as biological machineries for producing biomedical devices. Micro-structuration of BC's surface is possible using a Guided Assembly-Based Biolithography (GAB), which is a powerful replica molding methodology to transfer on-demand functional topographies to the surface of BC\(^20\,24\,25\). Thus, the formation of the pattern on the surface of BC inhibits the initial inflammatory response followed by a sequence of events that lead to the deposition of fibrotic tissue\(^8,19\). Such an event is correlated with several health risks for patients, both during control interventions and after, when the functionality of the implanted device is required\(^20\).

The intrinsic characteristics of BC, such as the non-toxic chemical composition, the purity, the high porosity, the bulk mechanical properties, and the matrix-like morphology, make it advantageous in biomedical applications to prevent the fibrotic adhesion, as documented by several studies\(^15,21–23\). Indeed, it was previously reported that the adverse conditions for adhesion, between cells and BC, are established by an isotropic distribution of topographical features, which physically interfere with the establishment and maturation of focal adhesion\(^21,24\). The micro-structuration of BC's surface is possible using a Guided Assembly-Based Biolithography (GAB), which is a powerful replica molding methodology to transfer on-demand functional topographies to the surface of BC nanofiber textures. BC nanofibers are assembled in a three-dimensional network reproducing the hexagonal symmetrical array on the surface were analysed. Promising outcomes have been obtained by 5 strains, which produced the required surface-microstructured BC. This study provides new evidence for the use of wild-type strains as native or functionalized biomaterial producers\(^1\).

### Results and discussion

**Assessment of bacterial cellulose production by Komagataeibacter strains.** The amount and shape of BC produced by AAB strains are presented in Table 1. AAB species considered for this study were *K. xylinus*, *K. hansenii*, and *K. sucrofermentans*, which are reported as the highest BC producers among the species of the *Komagataeibacter* genus\(^27–32\). BC was produced by all the strains; however, a great variability was observed not only on the macroscopic structure of the native BC (Supplementary Fig. S1), but also in terms of weight.
Some strains produced a uniform and homogenous BC layer, smooth on the surface, which was easily removed from the culture broth without damaging its shape. Whereas other strains produced a fragmented BC layer with a heterogeneous and compact macrostructure. Once removed from the culture broth the cellulosic matrix has lost its original shape.

Considering this study aimed to synthesize a medical device made of surface-microstructured BC, 9 strains producing uniform and smooth BC layers of different weights (Supplementary Fig. S2) were selected for further investigation. Among them, 5 strains were isolated from Kombucha tea, one from black cherry and 3 from unknown isolation sources (Table 1).

The variability in BC production has been previously observed for strains of the genus *Komagataeibacter* and within strains of the same species (e.g., *K. xylinus*). In the *Komagataeibacter* genus, differences in cellulose synthase (CS) complex have been correlated to a different ability in BC production. The main reason for this difference is due to the number of bcs operons, which generally span from 1 to 3 in members of the *Komagataeibacter* genus. However in *K. xylinus* species two strains were described to possess a fourth copy of bcsAB gene. We previously obtained and analysed K2G30 and K1G4 genomes. The K2G30 genome possesses three bcs operons and a fourth copy of bcsAB gene, that encodes the catalytic core of CS. Whereas K1G4 analysis revealed the presence of the two bcs operon types structurally completed and a third copy of bcsAB gene. These features can explain the high amount of BC produced by these two strains.

Other factors that contribute to strain variability in BC production are, the isolation source and handling of culture. Most of the strains of this study originated from food matrices, especially Kombucha tea, which is considered a selective source for the recovery of BC producing AAB, while others were originally collected from sugared and acidic products. The laboratory culturing of strains also affects the stability of phenotypic traits. This phenomenon is already observed for AAB when they are continuously cultivated and preserved by short-time preservation methods, which increase the formation of high rate of spontaneous mutants.

**Production of surface-structured bacterial cellulose with guided assembly-based biolithography.** Cultures derived from the nine selected strains were tested in the polydimethylsiloxane (PDMS) bioreactor, using the same conditions as during the previous tests (Hestrin–Schramm (HS) broth; 5% v/v inoculum; incubation at 28 °C for 7 days). Outcomes confirmed the great variability in native BC weight (Fig. 1a) (Supplementary Table S1) and characteristics. Some strains differed from the others to produce non-optimal BC pouches. K1G4, K2G30 and K2G39 strains produced the highest amount of BC, but the formed pouch did not have optimal attributes. Also, the visual analysis of transparency and thickness of the pouches varied among strains (Data not shown). To assess the suitability of the strains for the purpose of this study, it has been hypoth-

![Figure 1. Weight of native BC produced by screened strains. BC weight was obtained after incubation at 28 °C for 7 (a) and 3, 4, 5 (b) days inside bioreactor. Values are given as mean ± standard deviation (n = 3).](https://doi.org/10.1038/s41598-021-98705-2)
Table 2. Dried BC yield (g/L) produced after incubation at 28 °C for 7 days inside flasks and bioreactor. Values are given as mean ± standard deviation (n = 3). Different lowercase letters in the same column indicate significant differences (p < 0.05).

| Strain          | Flask (30 mL)   | Bioreactor (55 mL) |
|-----------------|-----------------|--------------------|
| K1G4            | 6.9867 ± 0.0033 | 3.7020 ± 0.4153    |
| K1G23           | 2.0967 ± 0.0033 | 2.8348 ± 0.2950    |
| K2G39           | 1.8011 ± 0.0069 | 3.8764 ± 0.4344    |
| K2G30           | 1.7311 ± 0.0051 | 4.8939 ± 0.2734    |
| K2A8            | 1.3367 ± 0.0033 | 2.5028 ± 0.5575    |
| DSM 15973T      | 0.5456 ± 0.0019 | 0.8077 ± 0.0387    |
| DSM 46604       | 0.5156 ± 0.0018 | 0.4907 ± 0.0621    |
| DSM 46590       | 0.8512 ± 0.0019 | 0.8721 ± 0.2112    |
| DSM 46591       | 0.6478 ± 0.0051 | 0.4992 ± 0.0705    |

Characterization of surface-structured bacterial cellulose with guided assembly-based bio-lithography. Since the surface micropattern designed by Hylomorph AG has characteristic dimensions in the sub-micron range (1–10 µm), brightfield microscopy (BF) and scanning electron microscopy (SEM) were used to investigate the presence of the surface micropattern on the pouches. All the 5 candidate strains (K2G30, K1G4, K2A8, DSM 15973T and DSM 46590) showed the hexagonal pattern on the surface using BF (Fig. 3a).

In parallel, SEM analysis with high magnification was performed to observe the surface of the matrix and to confirm the fibrous network of BC (Fig. 3b,c). Moreover, to visualize how bacterial cells are dispersed into the BC matrix (Fig. 3d), a K2A8 sample, as representative of the pool of strains, was differently treated for SEM experiment, by turning the BC pouch inside out and reducing the washing steps. Results are consistent with the literature describing AAB of Komagataeibacter genus as short rods with an average width of 0.65 µm (ranging...
from 0.5 to 0.8 µm) and an average length of 2 µm (ranging from 1.0 to 3.00 µm), occurring singly, in pairs or in chain51.

Conclusions
In this work, 25 Komagataeibacter strains were tested for producing a BC device suitable for biomedical purposes. The medical device was manufactured in the form of a pouch, synthesized by AAB at the liquid–air interface, after a period of incubation inside a PDMS bioreactor. Among studied strains, K2G30, K1G4, K2A8, DSM 15973T and DSM 46590 produced optimal surface-microstructured BC and they were designated as candidate strains for the purpose of this study. Although a further research step is required to evaluate the biocompatibility, the durability of the device and the reduction of fibrotic tissue, results of this study open new horizons toward applying wild-type AAB strains in the biomedical field.

Materials and methods

Materials. HS broth was prepared following the recipe: D-glucose 2% w/v, Yeast extract 1% w/v, Polypeptone 0.5% w/v, Disodium phosphate anhydrous (NaHPO4) 0.27% w/v, Citric acid 0.115% w/v) 44. Whereas the 105 broth was prepared according to DSMZ instructions: D-glucose 10% w/v, calcium carbonate (CaCO3) 2% w/v, Yeast extract 1% w/v. Sterilization was conducted in an autoclave at 121 °C for 20 min.

Bacterial strains and cultivation conditions. AAB strains used in this study were supplied by UMCC (Unimore Microbial Culture Collection, Italy) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) culture collections, respectively (Table 1). All the strains were handled by UMCC culture collection, according to the standard procedures of the Microbial Resource Research Infrastructure—Italian Joint Research Unit (MIRRI-IT)52.

DSMZ strains were revitalized according to manufacturer instructions, using 105 broth and UMCC strains on HS broth. After revitalization, a pre-inoculum was performed on 5 mL HS broth. Then an aliquot of inoculum (5% v/v) was transferred in a 100 mL Erlenmeyer flask containing 30 mL HS broth. Incubation was performed at 28 °C for 7 days, under static conditions.

Qualitative and quantitative tests of BC. BC production was qualitatively estimated following the method proposed by Navarro et al., 199953. Pellicle was collected from the broth culture after 7 days of incubation at 28 °C, treated with 4 mL of 5% NaOH and boiled for 2 h. BC production has been confirmed when the pellet did not dissolve after boiling. K. xylinus K2G30 was used as a positive control.
Estimation of BC yield was carried out following the method proposed by Hwang et al. 199954. Briefly, native BC from culture broth was collected and washed with distilled water four times with a time-lapse of 15 min and additional washing with 1 M NaOH. Washed BC films were kept at 90 °C for 30 min inside the solution of NaOH 1 M. Finally, BC was rinsed using distilled water four times and then dried at 25 °C until a constant weight was reached. The weighting of the dried BC film was performed using an analytical balance (Gibertini E42S, Milan Italy). The yield of BC was expressed as grams of dried BC per liter (g/L).

Growth of AAB in 3D bioreactors. 3D bioreactors manufactured in PDMS were used as vessels for assessing the development of surface microstructured BC by Komagataeibacter genus strains. Cultivation in the bioreactor was carried out with 55 mL of HS broth and 5% v/v inoculum. Bioreactors were covered with sterile gauze on the top and incubated at 28 °C for 7 days. Then, each sample was washed using at first distilled water, then a solution of NaOH 1 M and rinsed with distilled water several times. The last washing in distilled water was conducted in shaking conditions. BC production within the bioreactor was evaluated by the formation of a homogeneous film with a cap at an open liquid–air interface. The sealing of the pouch was removed aseptically and avoiding the damaging of the BC pouch-shaped device. The yield of BC produced was controlled as previously described and expressed in grams of dried BC per liter (g/L).

Microscopy. The surface microstructure of BC pouches was examined using a field emission scanning electron microscope (NovaNano SEM 450, FEI, USA). Samples were cut (5 × 5 mm²) and mounted on a stainless-steel stub with double-sided tape. The analysis was performed in a low vacuum mode (80 kPa) with an acceleration voltage of 10 kV55. The surface microstructure pattern of the films was obtained through a BF microscopy Eclipse Ts2 inverted microscope (Nikon, Tokyo, Japan).

Statistics analysis. The statistical analysis of the data was performed through analysis of variance (ANOVA) using multcompView package implemented in R v 4.0.456. The experiment was performed in 3 replicates. The differences between means were evaluated by Tukey HSD test (p < 0.05). The data were expressed as the mean ± standard deviation (SD).
Data availability
All data generated or analysed during this study are included in this published article (and its Supplementary Information file).

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Author contributions

M.B.: Methodology; Data curation, Writing original draft, Review & editing; F.R.: Methodology, Review & editing; S.L.: Methodology, Review; K.A.: Review & editing; H.H.: Methodology, Review; S.B.: Methodology, Review

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to A.F. or M.G.

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