Living Manufacture: Principles for a microbial 3D printer

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ABSTRACT: This paper will introduce the Living Manufacturing Project to develop a novel digital manufacturing approach by integrating biological growth and digital fabrication to make 3D functionally graded materials and objects. The paper will introduce this concept and initial experiments which connect the design of bacteria at the genetic level with the development of a future digital infrastructure. We see this work as the basis of a new fabrication technique with potential applications in a wide range of areas, including biomedical applications, complex composites for high-performance manufacturing and novel consumer products and the potential to scale-up to create novel materials and structures for architecture.

1 INTRODUCTION

The idea of growing or self-assembling a building using biological processes has been prominent in the speculative discourse in architecture. Biological systems exhibit the ability to assemble highly complex materials with little energy input. Furthermore, once these materials are constructed, they often continue to live, exhibiting functions such as self-repair and adaptation to the environment. The technical realities of a 'living architecture' seem some distance away and much speculation has been conducted without reference to scientific reality (Cogdell, 2019). Advances in the field of Engineered Living Materials (ELMs), however, are developing a scientific basis for this vision.

ELMs research uses a combination of approaches from Synthetic Biology and materials science to 'programme' cells as 'material factories' to synthesise materials intelligently and in response to environmental input. The ultimate goal of ELMs research is to develop synthetic biological systems which will operate in a similar way to a tree, where an instruction carrying seed can be planted in an appropriate environment and undergo all the developmental processes necessary to create a complex material structure using only the locally available resources.

ELMs for Architectural applications are still a nascent field. However, we suggest here that, well before we derive materials systems that are entirely self-assembled through biological processes, there is significant promise in approaches that combine techniques from digital fabrication with synthetic biology. Such novel fabrication techniques would make use of biology's capacity for efficient and complex material fabrication and our ability to influence these

processes through the precise spatial and temporal control possible with digital systems. We have previously described these processes as existing between a triad of *in silico* (in software), *in vitro* (through the control of environment often using computer hardware) and *in vivo* (in the living) (Dade-Robertson, 2020). This paper outlines the methodological approach of our *Living Manufacture* project which aims to create a novel fabrication system of microbial cellulose. In this paper, we focus preliminarily on the *in vivo* and *in vitro* experimentation as well as our approach to the design of a bioreactor-based '3D printer' as a fabrication system for bacterial cellulose. We will also briefly discuss the implications for the development of an integrated *in silico* system at the end.

1.1 *Bacterial Cellulose*

Cellulose is the most abundant biomaterial in the world and is already widely used in a range of manufacturing contexts. Estimates suggest cellulose production in the biosphere to be more than 7.5×10^{10} tons (Habibi, 2014). Pure cellulose is a linear polysaccharide made of 1,000 to 15,000 polymerised glucose monomers. Though conventionally, cellulose is commonly combined with other organic polymers such as lignin and hemicellulose to form commonly used biomaterials like wood. Bacterial cellulose (BC) is, in contrast, composed of 'pure' cellulose. Though many bacteria can produce cellulose, the *Komagataeibacter* genus is one of the most productive and can produce a pure BC pellicle. The cellulose-producing bacterium extrudes individual cellulosic fibres which are then aggregated by hydrogen bonds to form larger fibrils. The cellulosic fibrils can then interact with other fibrils to form a larger cellulosic matrix. Both hydrogen bonding and the matrix structure are crucial in imparting material strength to the BC. The BC material itself has high purity, crystallinity, tensile strength, biocompatibility and biodegradability (Klemm et al. 2001).

In recent years, BC has garnered much interest from different fields and has found applications in the field of apparel & design (Lee, 2012), food (Azeredo et al., 2019), biomedicine (Czaja et al., 2006), electronics (Ummartyotin et al., 2012), and bioremediation (Rezaee et al., 2005). Despite much interest from a variety of fields, current methods for BC fabrication are limited. BC pellicles are generally produced as a thick gelatinous mat which is subsequently dried to form a sheet. To derive more complex functions, forms, and structures BC requires further postproduction steps including purifying and reconstituting as a paste which can be extruded through a 3D printing process (Gutierrez et al., 2019). Ultimately, current fabrication methods using BC do not address the limitations of form and function limiting the potential applications of BC. Furthermore, given the complexity of cellulose composites we see in nature, the opportunities of using BC as the base material for more complex composite materials which could be grown for specific functional purposes.

1.2 *3D Printing*

Traditional 3D printing processes can be used to fabricate complex shapes using a range of materials and functionally graded microstructures of different densities. However, with these processes, there are limitations including a limited material pallet (Thomas-Seale et al., 2018), with materials that are difficult to biodegrade, challenges with multi-material printing and the high energy needed to work with the material, such as high heat for material melting (Annibaldi & Rotilio, 2019). Approaches have been developed which involve the printing of

living cells. These approaches largely come from tissue engineering and can inkjet or extrusion-based techniques (Bracaglia et al., 2017) where cells are encapsulated within a gel matrix or printed onto a scaffold structure. These techniques are however generally very small scale and for highly specialised medical applications.

With this project, we are investigating a new approach to 3D bioprinting which builds upon the ability to control microbial growth and maintain the complexity of the bacterial cellulose (BC) *in vivo* whilst also performing in growth modifications to the BC. Using this approach, we are currently developing a fabrication platform that will serve as a demonstrator for this approach in which 3D graded materials form by utilizing microbial growth and self-assembly of materials under digital direction with low (relative to traditional 3D printing) energy input and exhibit a degree of functional variability.

1.3 *Living Manufacture*

Living Manufacture project is a collaboration between bio-scientists and architects/designers to build a new type of EML based fabrication system to produce BC pellicles. Recent studies have demonstrated the potential of BC-based ELMs through genetically engineering BC producing bacteria and other microbes which are co-cultured with the BC-bacteria (Gilbert et al., 2021). By incorporating another engineered microbe (e.g., *Escherichia coli* or Yeasts) with the cellulose-producing bacteria, the engineered living BC material can respond to different stimuli (e.g. chemical and optical) and produce additional materials or modify the BC through, for example, the secretion of pigments or enzymes which will change the structure of the pellicle. The ability to induce these changes through engineered stimuli responses offers the possibility to control these modifications while the BC pellicle grows, thereby patterning the material in three dimensions. To achieve this, we are developing a fermentation vessel (Fig. 1). The design of these vessels is based on our preliminary proof-of-concept experiments to 1) increase the thickness of the cellulose and minimise the formation of separate layers, 2) co-culture with an engineered light-responsive (optogenetic) bacteria to trigger the production of a pigment and 3) weakens the material in specific locations using a cellulose-degrading enzyme. The project seeks to develop demonstrators for novel digital manufacturing approaches that integrate digital fabrication and biological growth to make 3D functionally graded complex materials and structures.

The methodological approach in this project is split into two parts: the first focuses on the *in vivo* processes of biological growth, establishing the optimum growing conditions for the BC in a modified environment as well as establishing compatibility with other engineered stains such as *E.coli* or yeast. The second part outlines the *in vitro* process through the control of the environment through the specification of the novel fabrication platform based on a modified lab liquid handling system. At the end of the paper, we will also discuss the potential to develop *in silico* process, defining the morphology and final modification pattern digitally and developing an integrated bio-digital fabrication system.

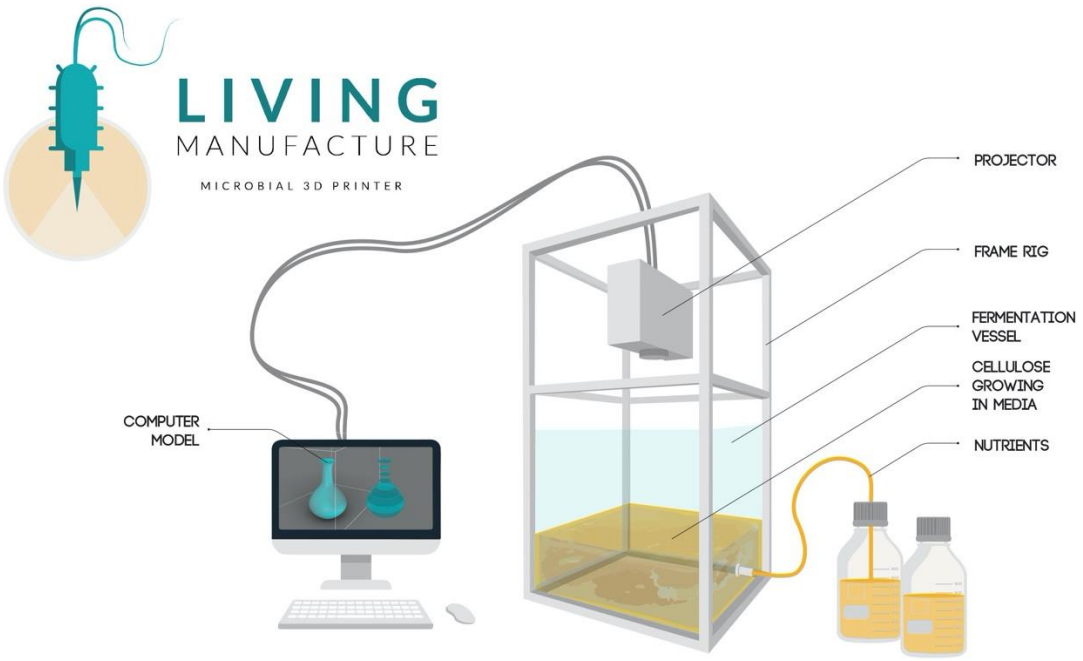


Figure 1. Concept design of bioreactor with digital and biological input.

2 IN-VIVO METHODS

2.1 *Optimal bacteria growth*

The methods utilized in this paper are based on existing protocols used to grow BC in Hestrin & Schramm's (1954) (HS) medium. *Komogataeibacter xylinus* DSM2325 bacteria used in this study was obtained from DSMZ culture collection. To obtain the BC pellicles, cultures were statically grown in the HS medium at 28 °C. BC production is usually based on the formation of a floating pellicle at the air-liquid interface in a glucose-rich medium. A variety of factors can influence a pellicle's thickness, these include access to nutrients for the most actively growing part of the BC (the top) and access to oxygen. This restricts the form of the cellulosic material to its culture vessel and results in a homogeneous thin-sheeted material once the pellicle has dried. The first set of experiments set out to test the limits of BC growth and maximize the thickness of the pellicle. These tests compared the influence of adding oil to the HS medium to increase the yield (Żywicka et al., 2018) and to eliminate the problem of creating separate cellulose layers.

2.2 *Optogenetic (light sensing) bacteria*

To engineer light responsiveness (optogenetics) into *Escherichia coli,* we genetically engineered the bacteria with optogenetic plasmids (which are rings of DNA that are found outside the bacteria chromosome) carrying the *el222*, the light-sensitive gene, and coding for a red fluorescent protein (RFP*)*, a gene for expressing a red fluorescent pigment, genes that were built and described by Jayaraman et al. (2016). We used two variants of the optogenetic system. In the first variant, a blue-light inducible variant (BLind) of the optogenetic system can produce a

red fluorescent protein (RFP) in the presence of light in *E. coli*. Thus, bacteria carrying this version of the plasmids would produce RFP pigment when exposed to light. In the second variant, a blue-light repressor (BLrep) can prevent RFP production in the presence of light and will only produce RFP in the absence of light. Thus, when exposed to light it would prevent the production of RFP pigments.

To co-culture both bacteria we ensured that both bacteria had similar bacterial populations. To achieve this *E. coli* was cultured overnight, while the slower growing *K. xylinus* were cultured for 3 days with HS medium containing cellulases. The presence of cellulases inhibits pellicle formation and results in a homogenous inoculant. Cellulases were then removed by washing the bacteria cells repeatedly. By strategically increasing the thickness through altering the chemical and physical space where the BC pellicle grows, we can produce the desired thickness in a relatively short amount of time as well as provide stability for any other interaction with the process without disturbing the growth. Our initial results indicate that a bond forms between the layers which produces a bonded increase in the thickness of the material (Fig. 2a). We were able to observe a change in pigment intensity in *E. coli* pBLind and *E. coli* pBLrep when exposing both engineered to strain to blue light. With *E. coli* pBLrep, we were able to note a 1.5-fold change in pigment when comparing dark to light incubation cultured in LB medium. Similarly, when pBLrep was cultured in HS, we were able to observe a similar fold change albeit a weaker signal. When observing pigment changes in *E. coli* pBLind, we observed an increase in pigment production when the bacteria is exposed to light relative to the unexposed cultures (Fig. 2b). And again, we were able to observe a similar response when culturing in HS medium. These results are consistent with those reported (Jayaraman et al., 2016).

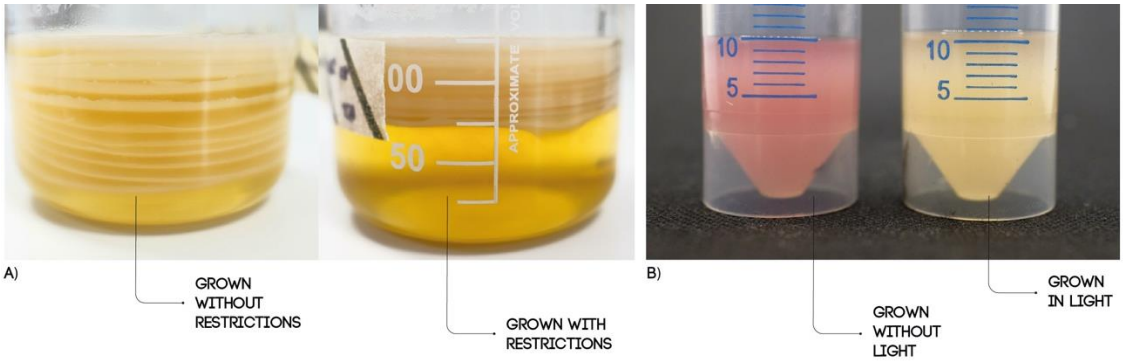


Figure 2. Results from increasing thickness comparing BC cultures grown as controls and where the layering has been minimised a). Results from the optogenetic pigment change b).

Once we proved the function of both optogenetic strains of *E. coli*, we investigated the viability of co-culturing the engineered *E. coli* with the cellulose-producing bacteria. When cultured together in an HS medium, both bacteria species remain viable. Moreover, both bacteria species remain functional in terms of their respective optogenetic and cellulosic capabilities.

3 IN-VITRO DESIGN

These *in vivo* experimental results inform the design of a bioreactor/fabrication of a vessel in which the cellulose growth can be optimised, and the light and chemical inputs can be programmed. Our design involves a bioreactor in which the pellicle will be grown by intermittently increasing the liquid levels (Hsieh et al., 2016) and replenishing the nutrients within a fermentation vessel. The first prototype aim will be achieved through a robotically controlled syringe head which will drip cellulase onto the surface of the pellicle. Where the cellulase is dripped we expect to see weaknesses in the material – weakening the cellulose structure. As shown in Figure 3, the cellulase is dripped into the growing pellicle as indicated by the 3D model, weakening the cellulose structure in those areas.

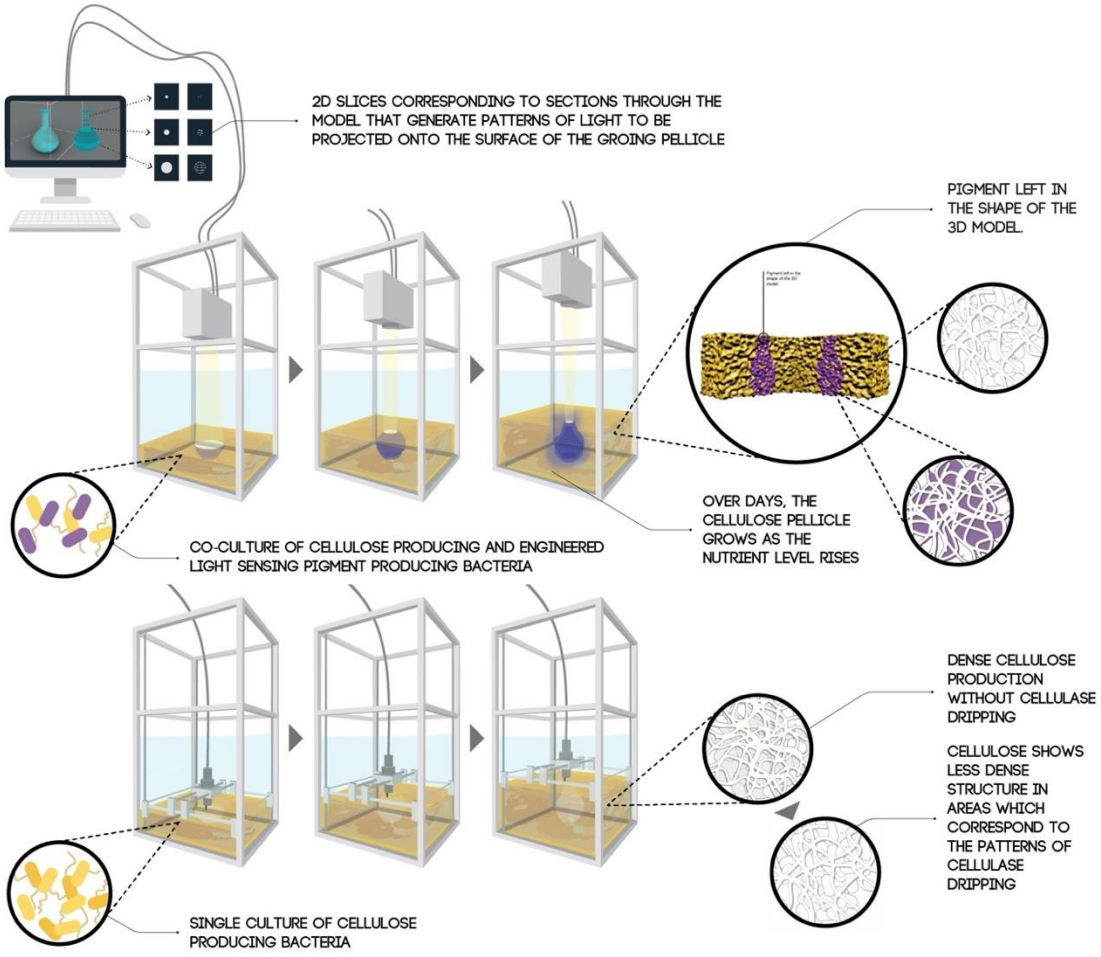


Figure 3. Diagrams showing the fabrication process in our two prototypes.

The second prototype will constitute a suspended projector within the fermentation vessel. The projector serves to project an image of the digital 3D model in slices that corresponds to the Z-axis (height) of the pellicle onto the growing pellicle in relation to the 3D model slides (Fig. 3). Similar to Stereolithography, the digital model is sliced into sections that are used to

expose the light-sensitive *E. coli* culture to different activation wavelengths of light. As the pellicle grows it will be modified by the light-sensitive bacterial cells that are embedded in the BC culture in the areas defined by the model.

The design and fabrication of the prototypes will be achieved through an open-source liquid handling robot 'EvoBot' (Faiña et al., 2020) that runs on an Arduino board to automate most of the culturing processes. The device input consists of a digital 3D model with information on the shape and distribution of materials. The design and functionality of the prototype shown in Figure 4 will be modified to accommodate the first prototype of this project. The robot will include processes that deposit a site-specific chemical to weaken certain parts of the BC material through automated liquid media feedings. As the BC pellicle is produced, sensors will be able to detect the location and raise the liquid medium level encouraging the pellicle to grow vertically in the vessel. The structure consists of a metal frame with the activation layer and syringe dispensers built using laser cut acrylic and Polyoxymethylene (POM) and 3D printed PLA parts. Making the construction of the set-up easy, relatively low cost and hackable for this project. There are elements that we still need to consider, including the chemical and light stimuli, such as sterilization of the environment and the controllable delivery of liquid nutrients.

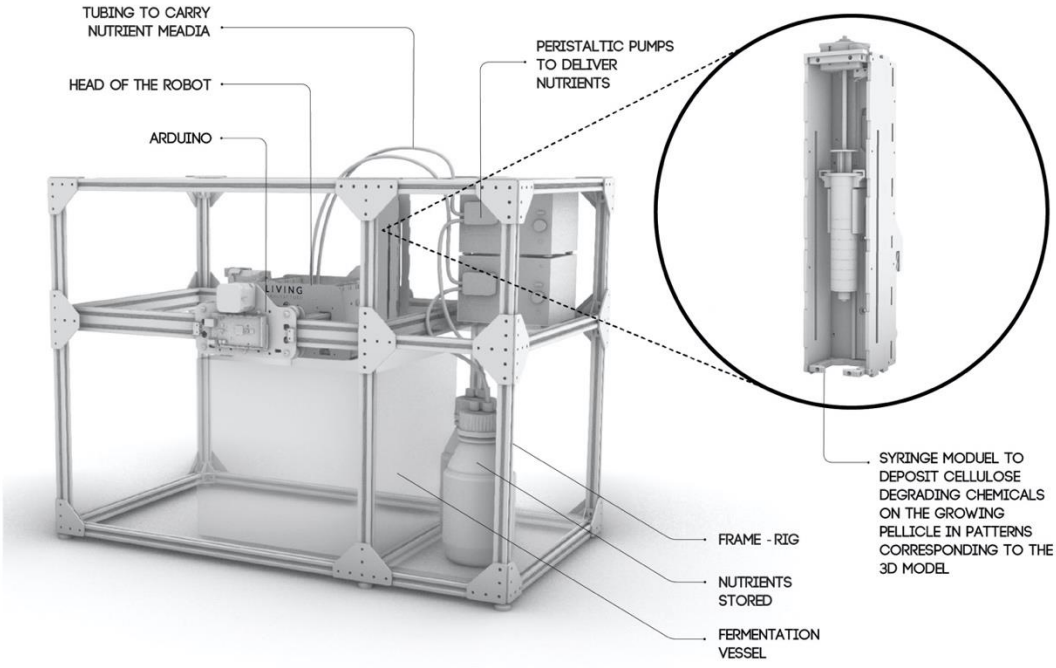
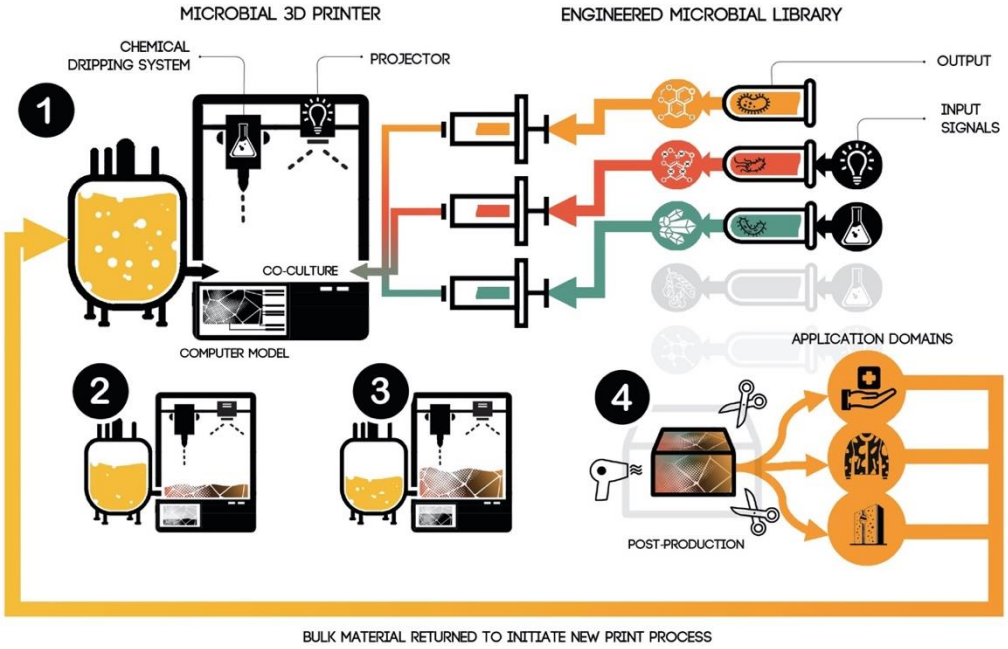


Figure 4. 3D model of the modular liquid handling robot, frame and head with the fermentation vessel below and a close-up of the syringe module.

4 DISCUSSION AND FUTURE WORK

With the Living Manufacture project, we are developing new types of fabrication infrastructure which aim to combine the energy efficiency and complexity of biological growth with digital

control. In our experiments, we focus on augmenting the material properties, not only through engineering the co-culture that can influence certain properties but materially engaging with the process in a designerly manner. This requires us to understand the limitations of the material *in vivo* and construct physical controls in the form of robotic manufacturing or 3D Printing processes *in vitro*.



A critical step to the integration of *in vitro* and *in vivo* fabrication will also require the addition of *in silico* sensing and control. Our prototype designs are posited on the idea that a computational model of a material pattern can be translated into patterns of light projected onto the surface of the growing pellicle and resulting in the production of key enzymes to modify a specific location on the cellulose. This will mean that our system needs to be able to monitor bacterial growth and the height of the pellicle adding additional nutrients – to maintain growth and control the relevant input for modification depending on the state of the pellicle over time.

Figure 5. Diagram to show the overall concept of a modular biological fabrication system with engineered bacterial co-cultured to produce different materials in response to inputs within a 3D printer like bioreactor. The outputs could be harvested and, in some cases, returned as food for new fabrication sessions.

We also believe that this novel system of fabrication is extensible in both scale and complexity. Pigment change, for example, could be seen as a stand-in for cellular processes which modify the cellulose or add in new materials, for example, other polymers or bio-minerals. This way the cellulose acts as a scaffold for a wide range of different cellulose composites materials,

including synthetic wood-like materials or alternatives to plastics, with different forms and functions. We envisage our prototype being developed as a modular like system with different compatible engineered microbial 'material factories' being plugged in like ink cartages and co-cultured within a bioreactor which can detect the microbe types and run the best program suited to their cultivation and the required material outcomes (Fig. 5). We view this kind of method as a pre-fabrication process, not in-situ construction, as the environment of this material development is highly susceptible to contamination. For now, these are contained lab experiments that adhere to UK regulation on safety to limit exposure and any potential risks to the environment posed by the engineered organism, which is classified under biosafety Level 1 which, if released outside the lab, could be viable in the surrounding environment but would have a negligible impact and could be easily managed. Additionally, these materials would be subjected to post-processing treatments before being taken out of that lab environment. Undoubtedly, in the long term, the safety of using engineered modified living cells need to be taken into consideration when this kind of fabrication process is developed. The advancements in cell-free synthetic biology offer opportunities to utilise the isolated cellular components and machinery to deploy the gene networks and metabolic pathways, rather than live whole- cells, in the fabrication processes. The cell-free system provides a potential mechanism for overcoming the risk raised from releasing genetically modified organisms into the environment. If this process can be scaled-up, we see potential in architecture but the most likely short- and medium-term applications will be in fields such as fashion and apparel and for medical applications. While there are no specific biological barriers to scaling up these processes for built environment applications, the process is still slow, with significant growth taking days and weeks. The pure bacterial cultures we use are also liable to contaminations which becomes a greater challenge the larger pellicles are grown. These challenges are not insurmountable but will be addressed as the research progresses.

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