Growing Fungi Structures in Space

Final Report

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1. Introduction

In order to increase the long-term mission sustainability it is necessary to use local resources to produce consumables, such as oxygen and water, and materials for construction. The value of an in situ resource utilization (ISRU) system is highest when the ratio between the mass of materials produced by the system and mass of the system itself, which has to be transported from Earth, is large. To that end, the investment in ISRU includes the costs of (a) prospecting to locate and validate the accessibility of indigenous resources, (b) developing and demonstrating capabilities to extract indigenous resources, (c) developing capabilities for processing indigenous resources to convert them to needed products, and (d) any ancillary requirements specifically dictated by use of ISRU [1].

To date many studies have shown that there are different resources available in the lunar and Martian regolith, which can be used for, for example, the production of surface habitats and infrastructure by various additive manufacturing technologies [2][3][4] [5][6][7][8]. Additive manufacturing technology is a very promising technique for utilizing in situ resources on the Moon and Mars. However, when using indigenous resources, it is important to consider the investments needed for (a) locating and validating the accessibility of indigenous resources, and (b) developing and demonstrating capabilities to extract indigenous resources, as mentioned before [1]. To bypass these requirements, in situ manufactured biocomposites might offer a cost effective alternative for the local construction materials. This would require transporting a minimal amounts (i.e. less than 1 mg) of fungal mycelium from Earth that could then be used as an inoculum for continuous in situ production of inocula. The inocula would be used with a local organic material to grow the biocomposite in-space. The production of biocomposite structures could be low cost with limited human assistance, eliminating therefore costly and time consuming processes of locating, validating and extracting of local resources.

Fungal biocomposites are composed of fungal mycelium and a plant waste substrate. Mycelium is a root network of fungus, a vegetative part which consists of thread-like hyphae. Fungus uses mycelium to absorb nutrients from the environment containing carbon and nitrogen in a two-step process. First, enzymes are secreted onto or into the food source by hyphae to break down biological polymers into smaller monomers. These monomers are then absorbed into the mycelium by diffusion and active transport. By consuming plant-based waste products, such as sawdust, mycelium's dense network binds the substrate into a structurally adequate material composite.

Fungal based biomaterials might offer the following advantages over other in situ manufacturing technologies:

1) Lower manufacturing and energy costs due to excluding the costs of (a) locating and validating the accessibility of indigenous resources, (b) developing and demonstrating capabilities to extract indigenous resources, and (c) developing capabilities for processing indigenous resources to convert them to needed products
2) Full manufacturing loop following a cradle-to-cradle principle: the waste of another process (e.g. organic matter from greenhouse) can be used as a basis for fabricating biocomposites, which at the end of their service can be biodegraded and used as a soil for plants
3) Light weight, therefore can be used for creating light weight structures
4) It is non-flammable and has good insulation properties
5) Enables to produce a large variety of materials from transparent films to leather and brick like materials
6) Allows designs with complex geometry

The main limitations are:

1. Needs special environment during the growth period (control of humidity, temperature, light, CO_2 and O_2, therefore energy is needed to sustain that environment)
2. Due to autonomous growing process of a biological material there is a factor of uncontrollability and uncertainty of the final material properties

2. Fungal biocomposites

The fungal diet is similar to that of animals and humans. Fungi use organic material as carbon source and convert it with oxygen to obtain energy. Moreover, they need a nitrogen, sulphur, and phosphor source, as well as micronutrients. They can extract these nutrients from organic waste streams such as plant material.

Filamentous fungi grow by means of filaments. These so-called hyphae have a diameter of up to 10 µm, a length of several cm, and form an interconnected network known as mycelium. Mycelia go easily undetected by eye when formed in soil or organic waste. Yet, they are very abundant in nature. One gram of soil can contain several km of hyphae. Mycelia can be seen with the naked eye when they grow into the air to form their fruiting bodies; the most conspicuous being the mushrooms. One should realize that the mushrooms represent only a minor fraction of the total biomass of the fungus.

Mushroom forming fungi can colonize large surfaces. For instance, an individual of *Armillaria bulbosa* had colonized an area >1000 hectares of forest making it the largest organism on earth [9]. Mycelia of many mushroom forming fungi such as *Schizophyllum commune* (*SC;* split gill fungus), *Pleurotus ostreatus* (*PO;* oyster mushroom) and *Trametes multicolor* (*TM;* turkey tail) feed on plant waste material such as wood, litter, and straw. They degrade the substrate while invading it with their hyphae. At the same time the hyphae bind the non-degraded material together.

Fig. 1. Pure mycelium (left) and biocomposite materials (right) by M. Montalti, Officina Corpuscoli

From a large group of fungi, Ascomycota and Basidiomycota are considered to be more suitable to create mycelium based materials as they can construct larger and more complex organic structures than other fungi. From the two, Basidiomycota have two important properties which can make them more suitable for producing biocomposites: septa and anastomosis. Septa, special transverse cell walls, have an opening that can be closed in order to block the draining of a cytoplasma through the rapture when hypha becomes damaged. This will decrease the damage of the colony and therefore will lead to faster colonization of a substrate. Also anastomosis increases the growth speed of mycelium by fusing two different hyphae together when they meet. In addition, it creates a more homogeneous network of mycelium which promotes a fast transport of nutrients [10].

Both, pure mycelium, as well as mycelium combined with a substrate, can be used as materials. The materials containing pure mycelium (Fig. 1, left) are created by allowing mycelium to completely

consume the substrate it is growing in. To that end, it is possible to fabricate rubber-like, paper-like, textile-like, leather-like, and wood-like material out of pure fungal mycelium by varying the environmental growth conditions, and physical and chemical treatments. The main limitation of fabricating materials out of pure mycelium is that the production process takes relatively long and that yield is relatively low, whereas the production process of fungal biocomposites (Fig. 1, right) by combining mycelium with substrate is fast and yield is high. In order to stop mycelium consuming the substrate completely the biocomposite needs to be treated at 60 ºC to kill the fungus. Depending on the fungus, plant waste substrate and physical and/ or chemical treatments it is possible to produced cardboard-like, softboard-like, hardboard-like, and brick-like biocomposite materials. Jones et al. [11] discuss the exact factors influencing the mechanical performance of the fungal materials, which are the hyphal architecture, cell wall composition, composite constituents and growth kinetics, in more detail. Also Haneef et al. [12] studied the ways to control and tune the physical properties of mycelium materials grown on cellulose and cellulose/ potato-dextrose. As the materials showed different relative concentrations in polysaccharides, lipids, proteins and chitin they concluded that these differences affected the morphology and mechanical properties of the materials.

2.1 State-of-the-art of fungal biocomposites for construction

One of the main benefits of biocomposites is that these materials are renewable and biodegradable, and therefore could be an interesting alternative to various plastics in packaging and insulating materials. To that end Holt et al. [13] developed and evaluated six blends of processed cotton plant biomass materials as a substrate for selected fungi for the fabrication of molded packaging material. They found that the developed biocomposite is a viable alternative to polystyrene foam packaging material by having similar mechanical properties. Jiang et al. [14] investigated a non-traditional approach for fabricating biocomposite sandwich structures based on natural textile fibre, mycelium-bound agricultural waste and bioresin matrix. The study showed that the strength of the panel depends on the type of substrate, degree of colonization within the sandwich skin by mycelium, and bonding between the core and skin. The stiffness was dominated by core strength depending on its thickness. Xing et al. [15] examined the thermal properties of mycelium based bricks as building insulation materials and concluded that the materials exhibited good thermal performance. Jones et al. [16] tested a number of mycelium based biocomposite materials for improved fire safety and found that the biocomposites are very economical alternative to highly flammable petroleum-derived and natural gas-derived synthetic polymers and engineered woods for insulation, furniture and panelling applications.

Fig. 2. ‘Hy-Fi’ by The Living, 2015 (left) and MycoTree by F. Heisel et al., 2017 (right)

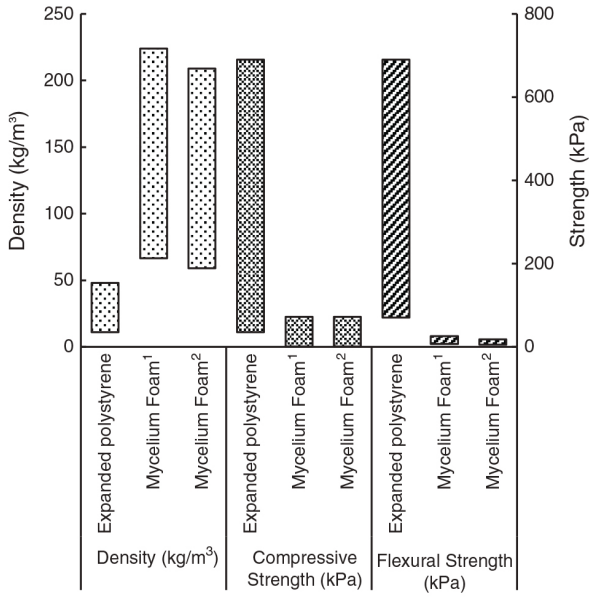


Fig. 3. Comparison polystyrene and mycelium foams, M. Jones et al., 2017

Although generally proposed as a potential replacement for polymer grade foams (Fig. 3), a number of studies have also investigated the application of fungal biocomposites in construction as bricks and boards (Fig. 4). The main physical requirement for a conventional building brick is its high compressive strength of around 8.6 to 17.2 MPa. To date there are no studies known where such high strengths have been achieved with fungal based materials, which normally have a compressive strength of around 0.5 MPa [11]. Travaglini et al. [17] suggested that natural foams can provide acceptable mechanical properties with the benefits of being lightweight, sustainable and inert. They modelled the mycelium composite in their study as an open-cell foam to assess the performance of the material. The results showed that the compressive strength of the material was almost three times as high as its tensile strength, which is common to open-cell foams. Therefore the mycelium biocomposite was closest to polystyrene foam based on its density and strength properties. Gonzales [18] investigated the possibilities of using the biocomposite material developed by Ecovative Design in creating architectural structures, such as shelters, by shifting the scale and modifying the composition of the material. New York based architecture studio The Living designed a structure for the MoMA competition using mycelium bricks (Fig. 2, left). The structural engineering of the temporary tower 'Hy-Fi' was carried out by Arup and structural testing at Columbia University [19]. The test results showed a very low elastic modulus with increased stiffness at very high stresses, meaning that the material acted more as a foam than a brick and therefore the structure had to be designed accordingly. Lelivelt et al. [20] evaluated the structural performance of different type of mycelium biocomposites. Although the compressive tests showed lower results compared to conventional structural materials, the biocomposites could still potentially offer an interesting alternative in construction when developed further. Heisel et al. [21] designed a load-bearing branching structure made of mycelium building elements (Fig. 2, right). Due to the conservative compressive capabilities of the material the optimal geometry of the structure was found through 3D graphic statics following the compressive loads only. Their study showed that it is possible to achieve structural stability when using weaker materials by utilizing clever geometry and therefore non-standard materials could be used as building materials.

Property	Unit	Standard particle board	MycoBoard™
Density	kg/m³	600–700	801
Flexural strength	MPa	14–18	15
Modulus of elasticity	MPa	2400–2800	2640

Property	Unit	Clay or shale brick	Mycelium brick[a]
Density	kg/m³	~1900	318
Compressive strength	MPa	8.6–17.2	0.5

Note: [a] *Ganoderma lucidum* on red oak biomass.

Fig. 4. Properties of mycelium bricks and boards, M. Jones et al., 2017

3. Growing fungi structures in space environment

One of the show stoppers for the survival of biological organisms in space is the exposure to high radiation levels, such as galactic cosmic radiation (GCR), solar winds and solar particle events (SPEs). Therefore, it is necessary to evaluate whether the chosen model organism is able to survive in such an

environment. There is evidence that a specific type of fungi can survive the simulated Martian conditions [22][23] and that the ionizing radiation can even enhance the growth of melanised black fungi [24][25][26]. Onofri, de Vera, Zucconi, et al proved in their Lichens and Fungi Experiment (LIFE) that *Cryomyces antarcticus* and *Cryomyces minteri* are able to survive the simulated martian conditions aboard the Internatinal Space Station for 18 months. They found that more than 60% of the cells and rock communities did not undergo any change due to the exposure [23]. Dadachova, Bryan, Huang, et al studied melanised microorganisms, such as *Cryptococcus neoformans*, *Wangiella dermatitidis* and *Cladosporium sphaerospermum* and found that ionizing radiation changes the electronic properties of the organisms and enhances their growth [24]. In another study, researchers were able to provide clues how melanised black yeast *Wangiella dermatitidis* has adapted the ability to survive or even benefit from exposure to ionizing radiation [23].

3.1 In-space cultivation of organic substrate

To date most fungal biocomposites were made out of higher plants, such as straw and saw dust, as a substrate. However, cultivating higher plants in space is expected to be complicated. Here, mycelium was combined with a lower plant, the aquatic fern *Azolla filiculoides* (AF). *Azolla* is a fern, up to 2.5 cm tall, which floats on water either individually or in mats up to 20 cm thick. It grows in ponds, ditches, water reservoirs, wetlands, channels and slow flowing rivers and therefore does not need soil [27]. A unique property of the genus *Azolla* is its symbiotic relationship with the nitrogen-fixing blue-green alga *Anabaena azollae*. Due to its symbiotic co-evolution with *A. azollae*, the floating fern is able to absorb its nitrogen from air and therefore grow on nitrogen-deficient matter, which makes its cultivation in space less complex. Another benefit is its high distribution rate by being able to double its area in only 7 to 10 days under suitable conditions. AF can also be used as food and feed due to its protein content, as fertilizer for plants and for the biofuel production.
For the cultivation of AF in space a controlled environment has to be created (Fig. 5). AF is usually grown in nursery beds of 3 x 4 m and 10 cm deep, in groups of ten or twenty. The atmosphere in the controlled environment has to contain CO_2 and N_2 as the plant needs it for the survival. As the O_2 is released by plant due to the process of photosynthesis the controlled environment should be part of a closed loop system for the gas exchange between the living environment of astronauts and nursery of AF. Also other aspects of the environment have to be controlled, such as the temperature between 15 °C and 26 °C, pH of water between 4.5 to 7, light intensity between 20 000 to 80 000 lux, nutrient composition and humidity [28].

3.2 In-space cultivation of mycelium

In-space manufacturing of vegetative mycelium, called spawn, would also require a controlled environment (Fig. 5). Typically, the process starts with the preparation of pure culture from mushroom spores or tissues. The culture is then added to a nutrient-rich agar or sugar-rich liquid broth on a petri dish. Depending on the type of fungus, after 7 to 21 days of incubation period at 25 °C ± 2 °C, the mycelium has grown out. A part of that mycelium is then transferred to a container filled with cooked and sterilized grains for the production of mother spawn. In our case, the grains would be replaced with the biomass from *Azolla*. The colonization of that biomass would happen after 10 to 21 days of incubation period at 25 °C in dark. The mother spawn can then be used for the further inoculation of the substrate used to prepare the feedstock for 3D printing structural elements [29].

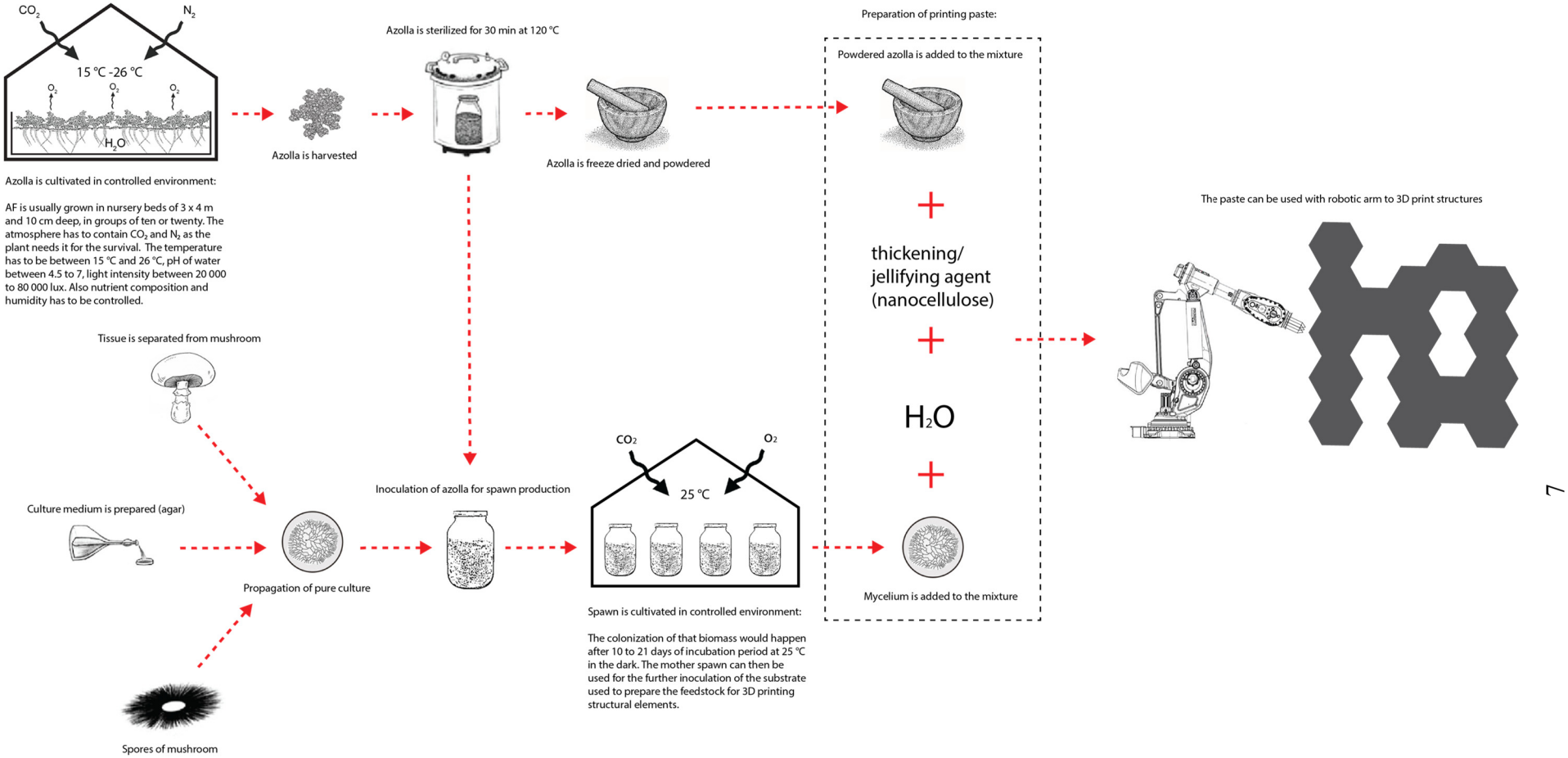


Fig. 5. Schematic of proposed in-situ manufacturing process of fungal biocomposite paste for 3D printing, H. Läkk, 2018

4. Original workplan

The work packages aim to modify tyrosinase gene expression to constitutively produce melanin in a mushroom forming fungus (**WP1**), match fungal strains with *Azolla* substrates (**WP2**), modify the properties of the mycelium materials (**WP3**), and assess the properties of the novel fungal based bio-composites (**WP4**). We will use the fungi *SC, PO,* and *TM* and the fern *AF*. Fern biomass will be produced in the facilities of the Molecular Plant Physiology group of Utrecht University, while the biocomposites will be produced within the facilities of the Microbiology group. Microgravity experiments and characterization of fungal biocomposites will be performed at ESTEC.

- **WP1. Constitutive melanin production**
  The tyrosinase gene of *SC* (fgenesh1_pg.9_#_454) is only expressed in its mushrooms. We will clone this gene behind the highly active constitutive tubulin promoter in a vector with the nourseotricin resistance cassette. Introduction of this construct in *SC* [30] should give rise to a mycelium that produces melanin. The melanin would not only protect the fungus while growing in space but would also protect the fungal bio-composite from deterioration under extreme space conditions.

- **WP2. Matching fungal strains with substrates**
  SC (either or not melanized)*, PO,* and *TM* will be grown in 1-4 cm thick layers of *AF* biomass. *AF* biomass will be used directly or after extraction of protein that could serve as animal or human food. Substrate will be inoculated with spawn (wheat grains colonized with mycelium) and grown for 1-4 weeks at 25 °C. Biomass formation under normal and micro-gravity will be monitored.

- **WP3. Modification of mycelium materials**
  SC (either or not melanized)*, PO,* and *TM* will be grown on *AF* biomass at different CO_2 and O_2 levels in the light or in the dark in 1-4 cm thick layers for 4-21 days. Materials obtained could either be used in space directly or after heat or cold pressing without human assistance. Possibly chemical treatment (e.g. with 1-5 M HCl, 1-5 M citric acid, 1-5 M NaOH, 1-4% formaldehyde, 1-10% glyoxal, and / or 10-40% glycerol) is needed to meet requirements for use of bio-composites in space.

- **WP4. Properties of the novel fungal based bio-composites**
  Properties of the bio-composites made in **WP2** and **WP3** will be compared with those based on straw and saw dust. To this end, we will perform thermal testing as well as testing of compression strength, stiffness, elasticity, air permeability, and behavior in vacuum, low- or hyper-gravity. Testing under low and high radiation will be part of a follow up project in case this project is successful.

5. Results

WP1. Constitutive melanin production

The *SC* tyrosinase gene (fgenesh1_pg.9_#_454) was cloned behind the highly active tubulin promoter in a vector with the nourseothricin resistance cassette. Transformants did not produce more melanin

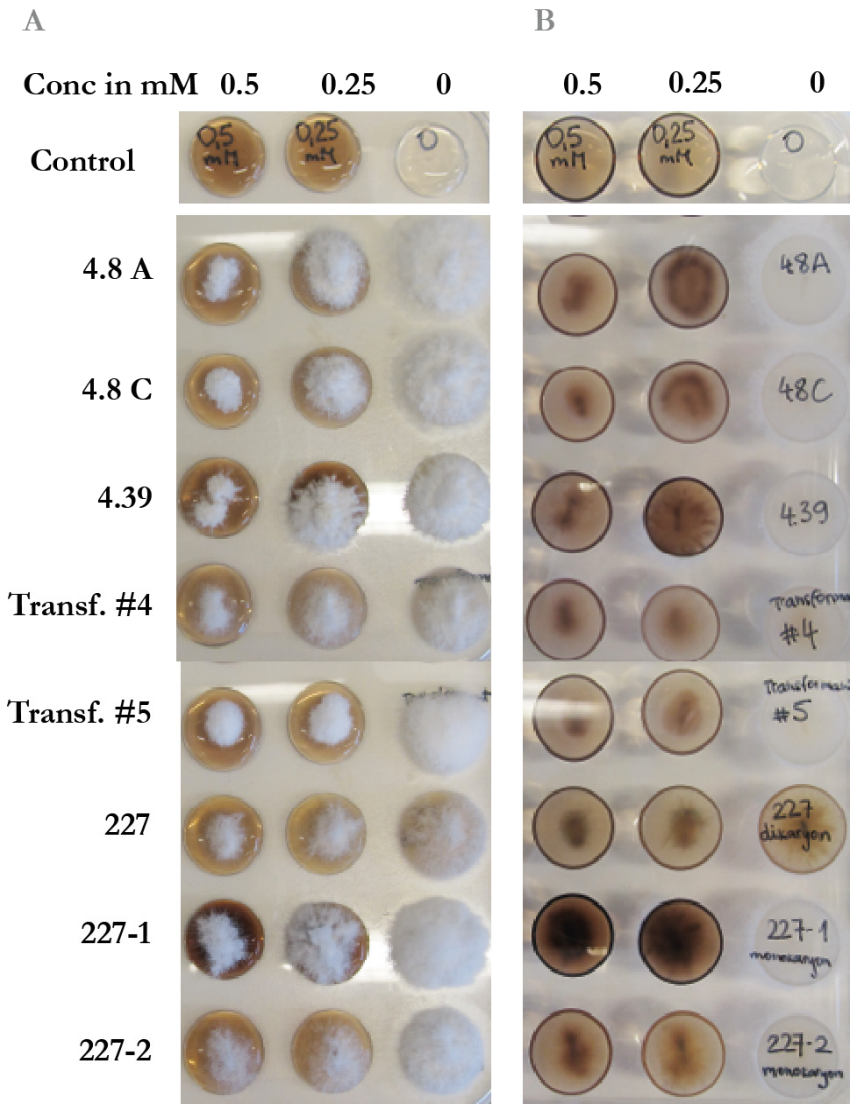


Fig. 6. Melanin production in wild-type strains 4.8A, 4.8C, 4.39, 227, 227.1, 227.2 and transformants #4 and #5

when compared to the wild-type strains. Apparently, SC uses other genes to produce melanin. As an alternative, we assessed whether it would be possible to induce *SC* melanin production by adding the precursors L-dopa (not shown) and catechol to the medium (Fig. 6). The optimal concentration of both substrates was 0.25 mM with higher concentrations inhibiting growth. Notably, we observed that strain 4.39 produced more melanin when compared to strain 4.8 strain. This prompted us to screen our natural strain collection. The natural dikaryotic *SC* strain 227 did not form more melanin when compared to 4.8 and 4.39 strains in the presence of melanin precurors. Notably, it did form melanin in the absence of catechol, which was not observed in the case of 4.39 and 4.8. Also of interest, monokaryon 227.1 (derived from strain 227) produced most melanin in the presence of catechol (Fig. 6). Together, 227 and 227.1 were the candidates of choice for growth on *AF*.

WP2. Matching fungal strains with substrates

AF was grown on minimal medium (0.7 mM KNO_3, 0.1 mM $Ca(NO_3)_2$, 0.13 mM KH_2PO_4, 0.1 mM $MgSO_4 \cdot 7H_2O$, 4.7 µM Fe-EDTA, 2.2 µM $MnSO_4 \cdot H_2O$, 0.1 µM $Na_2Mo_4 \cdot 2H_2O$, 8.1 µM H_3B_3, 0.06 µM $CuSO_4 \cdot 5H_2O$, and 3.1 µM $ZnSO_4 \cdot 5H_2O$) refreshing nutrients every week. One third of the biomass of each container (30 x 50 cm) was harvested each week, sterilized for 30 min at 120 °C, and freeze dried resulting in 3.8 g dry weight plant material per container (i.e. a total of 30 g dry weight out of 8 containers on a weekly basis). *SC* strain 4.8A, *PO* strain PC9, and *TM* strain X were grown on *AF*, either or not homogenized by grinding under liquid nitrogen (Fig. 7). Water was added resulting in 12.5 % - 33.3% *AF* w/v. After 7 days *SC* had formed most biomass in the 9 cm Petri dish (Fig. 7B), while TM had stopped growing after 3 - 4 days. Strains were also grown on 33% and 5% *AF* powder (w/v) agar (1.5%) plates. Also in this case, *SC* had most strongly colonized the substrate after 5 days (data not shown). Notably, 227 showed stronger growth when compared to 4.39 and 4.8A. Again, this shows that the latter strain is the preferred strain for forming biocomposites.

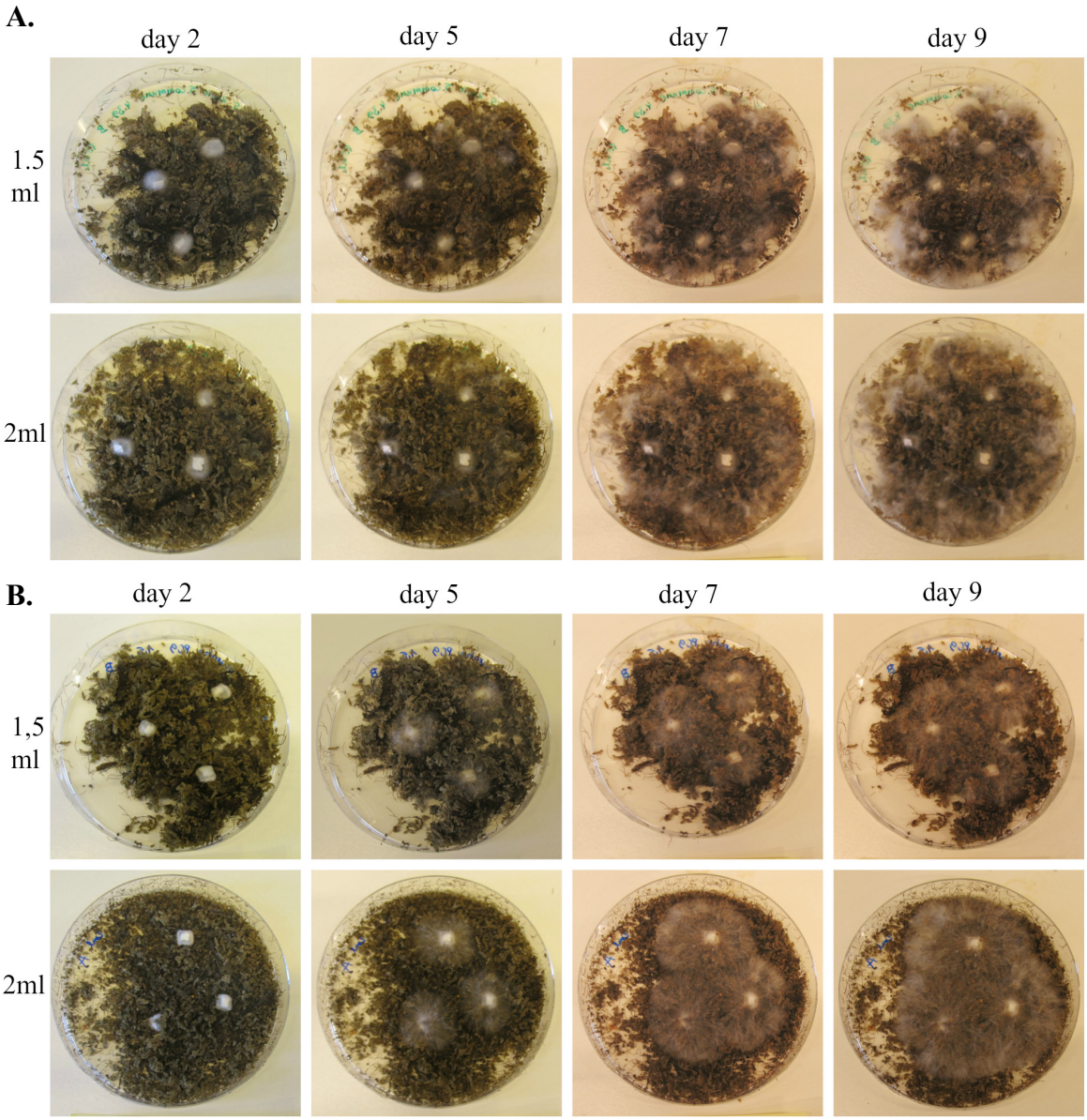


Fig. 7. Growth of *PO* (A) and *SC* (B) on freeze dried *AF* in 9 cm Petri dishes (point inoculum)

WP3. Modification of mycelium materials

Large Scale Growing of SC 227 on AF

To upscale growth of 227 on *AF*, we grew the fern in daylight in a plastic pool with a volume of 3000 L inside a greenhouse (Botanical Gardens, Utrecht University). Air flow in the medium was provided with an aquarium air stone set at regular time intervals. Every week, 33% of the plants were harvested followed by the addition of agricultural quality KH_2PO_4 (123 g/l), K_2SO_4 (150 g/l), $MgSO_4$ 7·H2O (250 g/l), Fe-DTPA (100 mmol), $CaCl_2$ 2·H2O (400 g/l), $MnSO_4$ (4 g/l), H_2BO_3 (5.4 g/l), $ZnSO_4$ 7·H2O (1 g/l), CuSO4 5·H2O (0.156 g/l), and Na_2MoO_4 2·H_2O (0.252 g/l). The water and nutrients were completely refreshed once a month. Harvested *AF* was air-dried in a fume hood or stored in a freezer and dried by freeze drying. 60 kg of wet weight *AF* resulted in 3 kg dry weight material. Several prototypes were made by growing *SC* on *AF*. To this end, 1 volume of water was added to *AF* ground under liquid nitrogen, followed by sterilization at 121 °C for 20 minutes. *SC* 227 spawn was used to inoculate *AF* and pre-grown at 25 °C for 7 days. The mixture was transferred to plastic moulds to prepare

the samples for mechanical testing after adding 10% sterile psyllium, and pressed by hand (Fig. 8, left). Growth was prolonged for ≥ 7 days in the dark while covered with cellophane. Mechanical pressing was unsuccessful and resulted in a very brittle material (not shown). During drying of the colonized substrate the composite bended and shrunk (shrinkage in width, length, height, volume: 14%, 13%, 33 %, 49,5%). Drying in a vacuum oven (ESTEC) did only partly prevent bending of the material (not shown). Bending was abolished by drying gradually in a circulated fumehood using a weight and metal grid. Still, materials were brittle and cracked during drying. Cracking was not observed when we used *Azolla caroliniana* as substrate (Aquaplantsonline, The Netherlands) (note PCR indicated that this species was in fact *Azolla cristata*). This is probably due to the lower content of phenolic material enabling more efficient colonization, thereby creating a more homogenous material (Fig. 8, right).

Fig. 8. Preparation of a sample in a plastic mould (left) and finished prototype of *AF* and *SC* composite after waterjet cutting (right)

Additional WP. Space environment effects on fungal growth

Growth of SC under simulated microgravity conditions

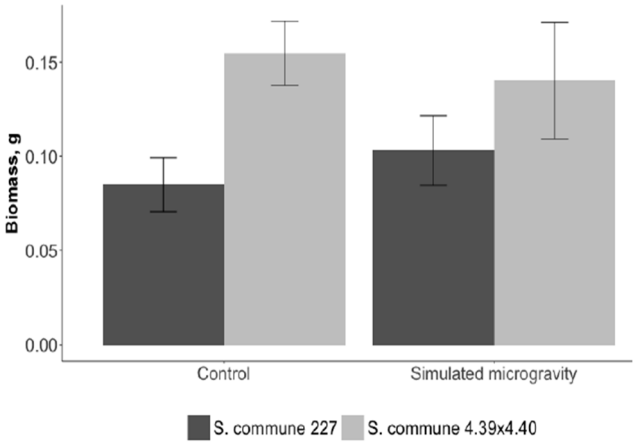


Fig. 9. Effect of simulated microgravity on the biomass of SC 4.39x4.40 and 227 strains (including the weight of the PC membrane)

Microgravity was simulated using a random positioning machine (RPM) [31]. *SC* strains were grown on 20 ml SCMM plates on a perforated poly-carbonate (PC) membrane (0.1 µm pores, 76 mm diameter; Profiltra, Almere). Plates were co-inoculated with 4.39 and 4.40 and 227.1 and 227.2 to enable mating. Cultures were pre-grown at 25 °C in the dark for 4-5 days and transported to ESTEC. Samples (biological triplicates) were placed in the RPM for 3 days, after which dry weight biomass was determined (Fig. 9). 4.39x4.40 showed no significant change in biomass formation in simulated microgravity, while 227 was shown to grow faster in RPM conditions ($p = 0{,}0131$).

Growth of *SC* exposed to radiation

Table 1. Colony forming units (CFU) resulting from 0.04 and 0.004 g *SC* mycelial macerate after irradiation.

Strain	Irradiation (Gy)	CFU (0,04 gr inoculum)	CFU (0,004 gr inoculum)
439x440	0	606-800	30-119
439x440	20	504	32-56
439x440	200	130-323	15-47
227	20	10	2-4
227	200	52	2

SC dikaryons 4.39x4.40 and 227 were grown for 6 days at 25 °C in the dark on 20 ml SCMM plates. These cultures were irradiated at ESTEC with 0, 20, or 200 Gy Co-60 for 3 days. The colonies were macerated in 50 ml SCMM for 30 seconds at low speed. 0.04 g and 0.004 g of mycelium was plated and colony forming units (CFU) were determined after 3 days of growth at 30 °C in the dark (Table 1). Both strains survived 200 Gy (60 x the lethal dose for humans [32]) although viability of 439x440 was 3-fold lower when compared to untreated mycelium (Table 1). To assess whether melanin production is induced by radiation the spent medium was set to pH 10 with 1 M NaOH, sterilized at 121°C for 20 min, and centrifuged for 5 min at 6000g at 4°C. The pH of the supernatant was set at pH 2 with 18.5% HCl to precipitate melanin. After 48 hours of incubation at 4 °C, the melanin was pelleted by centrifuging 10 min at 6000g at 4°C, followed by washing with water, chloroform, ethyl acetate, and ethanol [33]. Samples were washed 3 more times with water and dissolved in 1 ml 50 mM borate buffer (pH 8.0) followed by quantification of melanin at 200 – 800 nm using a DU-800 UV/VIS spectrophotometer (Beckman Coulter). Results showed no differences between non-irradiated and irradiated cultures, showing that melanin production is not induced by gamma rays.

WP4. Properties of the novel fungal based bio-composites

The material samples have been sent to ESTEC. The mechanical tests are planned in the near future.

WP5. 3D printing of biocomposite

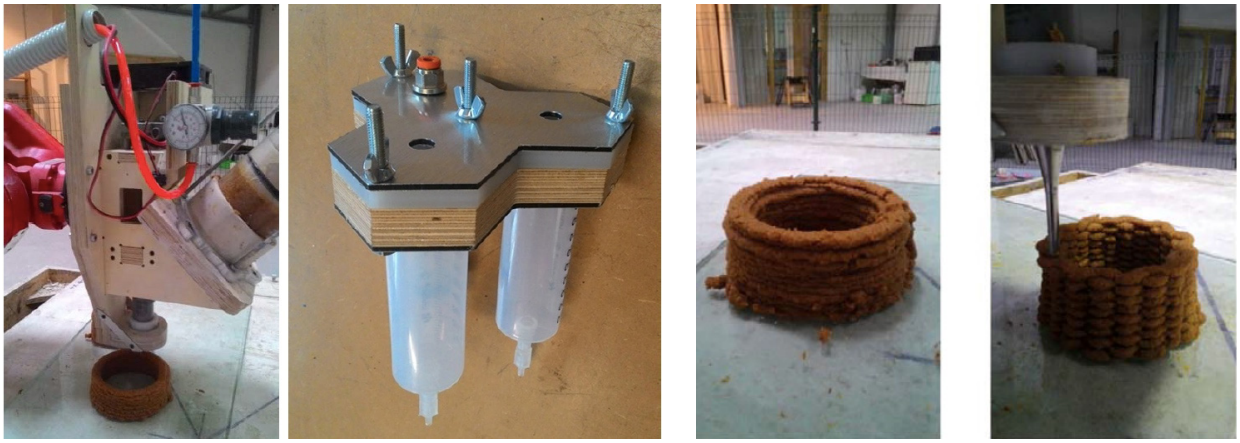


Fig. 10. Bio-material deposition using the paste material extruder I (1st, left) or the water-based extruder II (2nd, left). 3D printing test and objects (right), Officina Corpuscoli, Co-de-iT, digifabTURINg, 2017

Two typologies of extruding tools have been developed, suitable for being mounted on a robotic arm and specifically addressing the deposition of biomaterials (collaboration Officina Corpuscoli, Co-de-iT & digifabTURINg). Extruder I (Fig. 10, left) has been designed to deposit a mixture of mycelium, plant matter, and additives (thickening agents and/or jellifying substances). It is pneumatically and mechanically activated, is able to start and stop extrusion at will, and speed and air pressure can be varied. The material, contained in a 2500 cm^3 tank is transported by air pressure in a Y pipe where a plastic screw, connected to a servo motor, pushes the material through a customized nozzle (with sizes ranging from 3 mm to 18 mm). Extruder II (Fig. 10, left) that is currently under development has been specifically designed for extrusion and deposition of hydrogels. A pneumatic system controls the pressure needed to activate 3 different syringes, each enabling simultaneous deposition of a component (e.g. fungus, substrate, hydrogel).

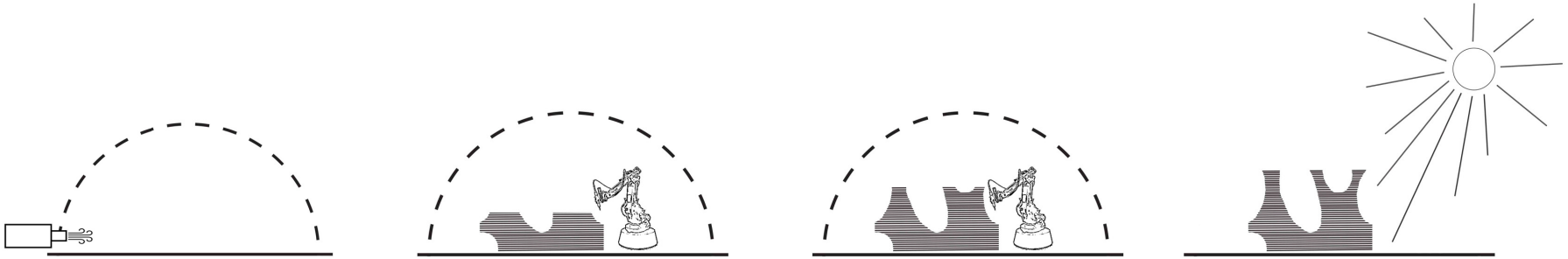
Fig. 11. 3D printed objects in different stages of colonization: just printed, after 3 days and after 1 week of growth, Officina Corpuscoli, Co-de-iT, digifabTURINg, 2017

The custom-made Grasshopper® algorithm in combination with an electronic control system controls which material/syringe to activate and at which deposition speed. The computational design approach allows to digitally approximate, simulate, and render a preview of the material deposition process. Parameters involved are related to the material (i.e. type of biomaterial, density and humidity), the robot (arm speed, deposition layer thickness, end-effector spatial orientation) and the extruder (extrusion speed, deposition start and stop). Tests have been performed to identify the optimal composition of the substrates for 3D printing, and fungal growth. Sawdust has been selected as the model substrate because of previous experience and the fact that particle size can be adapted. PO and TM were selected as fungal models and agar, alginate, corn starch, guar gum, arabic gum, and psyllium (and combinations thereof) have been used as thickening and/or jellifying agents. So far, the combination of saw dust (68%), psyllium (8%), and water (24%) was most successful for printing and fungal growth (Fig. 10, right, and 11).

6. Concept for robotic manufacturing of biocomposite structures

The proposed manufacturing sequence includes four phases (Fig. 12):

1) A temporary inflatable structure is installed with the specific internal environment needed for fungal growth
2) A robotic arm is placed inside the inflatable to print the structure
3) After printing, the system is left untouched until the mycelium has fully grown through the substrate to form biocomposite
4) The growth of mycelium is stopped by removing the temporary inflatable structure and exposing it to very low or very high temperatures



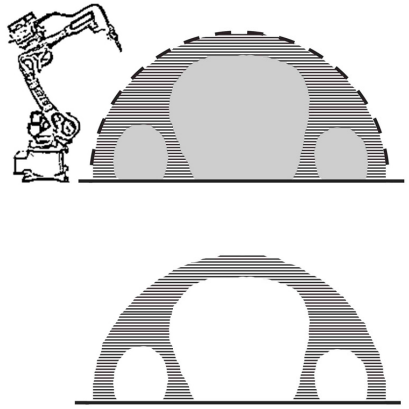
1. A temporary inflatable structure is installed with the specific internal environment needed for growing mycelium structures

2. A robotic arm is placed inside the inflatable to print the structure

3. After printing, the system is left untouched until the mycelium has fully grown through the structure to form a biocomposite

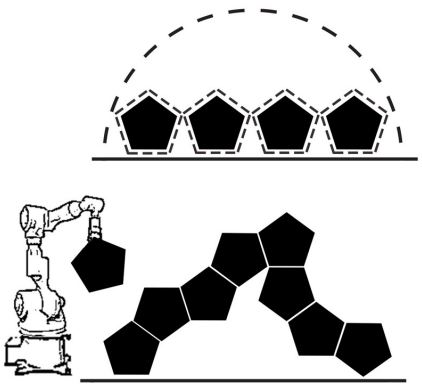
4. The growth of mycelium is stopped by removing the temporary inflatable structure and exposing it to very low or very high temperatures

OPTION 1



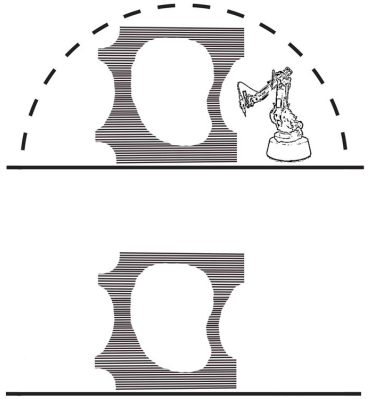
The temporary inflatable structure acts as a mold to shape the 3D printed biocomposite. In the same printing process, a supporting structure (gray) is also printed using a substrate made from some other material than the biocomposite. When the biocomposite is fully grown, the inflatable structure is removed and the biocomposite is exposed to extreme temperatures to stop the mycelium growth. The supporting material is also removed to reveal the required form. Also additional infltables can be used for the internal forwork instead.

OPTION 2



The temporary inflatable structure acts as a workshop for the production of biocomposite elements, such as building blocks or furniture. The elements can take any necessary form based on the specific mold applied. The inflatable structure ensures the right environmental conditions for the continuous production of the elements. The finished building elements can be removed from the molds and put into place using a robotic arm.

OPTION 3



The temporary inflatable structure provides the necessary environment for the 3D printing of a biocomposite structure. In this case the viscosity of the printed substrate is thick enough to be printed without a mold and supporting materials. Therefore many different shapes can be produced with the help of a robotic arm. After the completion of the printing process the structure is left untouched until the mycellium has grown through the substrate. The growth is stopped by removing the inflatable structure.

Fig. 12. Proposed sequence for in-situ robotic manufacturing of fungal biocomposite structures, H. Läkk, 2018

Based on the capabilities of the developed printing paste two options for the fabrication of biocomposite structures can be envisaged:

1. In the first case a temporary inflatable structure is installed to create the necessary conditions for the fungal growth and for controlling the biocomposite shape. Together with the biocomposite also a support structure is printed using suitable material for that specific function. When the biocomposite is fully grown the inflatable structure is removed exposing the biocomposite to extreme temperatures and stopping the fungal growth. The supporting material is removed to reveal the final form of the habitat structure.

2. In the second case the temporary inflatable structure acts as a workshop for the production of biocomposite elements, such as bricks or panels. The elements can take any necessary form based on the specific mould applied. The inflatable structure ensures the right environmental conditions for the continuous production of components for the construction. The finished building elements are then assembled into bigger structures by using robotic arm.

3. In the third case the temporary inflatable structure provides the necessary environment for the 3D printing of a biocomposite structure. In this case the viscosity of the printed substrate is thick enough to be printed without a mould and supporting materials. Therefore a variety of complex shapes can be produced with the help of a robotic extruder. After the completion of the printing process the structure is left untouched until the mycelium has grown through the substrate. The growth is stopped by removing the inflatable structure.

7. Next steps

One of the following steps of the study is to evaluate mechanical properties of the developed material. Therefore compression and 4-point bending tests are planned at the facilities of ESTEC in the near future. In addition, further steps have to be taken to improve the mechanical properties of the material by possibly reinforcing it with fibres or combining the biocomposite with other suitable additives. The developments in 3D printing study aim to evaluate the *Azolla* based printing paste for its printing and structural properties. If successful, tests on larger scale structures need to be conducted.

8. Conclusions

Fungal based biocomposite materials might offer a cost-effective alternative to in-situ manufacturing of habitat structures and elements based on indigenous resources, mainly due to the lower manufacturing costs, recyclability and light weight. Many studies about terrestrial applications of fungal biocomposites have already shown the possibilities of using the material in construction. Although, at the current state with much weaker mechanical properties than conventional building materials, it has successfully been demonstrated that the material can be used in load-bearing structures when structurally optimised to compression strengths only. Future developments in the field of biocomposites promise improvements in the mechanical properties of the material, therefore opening up new areas for the application possibilities.

In our study we aimed to study the feasibility of using biocomposite material for space applications. We evaluated the possibilities of in-space manufacturing of newly developed fungal biocomposite material by conducting preliminary experiments on matching plant based substrate with fungus, effects of space environment on fungal growth and 3d printing of biocomposite structures. To that end we were

able to identify the best performing fungus that grows on *Azolla filiculoides* (AF) substrate. We found that *Schizophyllum commune* (SC) grows better on AF when compared to *Pleurotus ostreatus* and *Trametes multicolor*. Tests showed that growth is possible at micro-gravity conditions and growth of SC may even be stimulated by micro-gravity. We were able to confirm that growth is possible at high radiation levels. SC was able to survive even at 200 Gy dose level of gamma radiation for 3 days. In addition, small scale AF/SC biocomposite panels have been produced for the mechanical testing at ESTEC. In the framework of 3d printing study tools for extrusion of paste-like biocomposites have been developed, computational design strategies have been implemented for controlling robotic devices and for consequent deposition of mycelium based pastes and different shaped 3D printed mycelium composite objects have been created.

DELIVERABLES

- Identification of the best performing fungus; *SC* grows better on *AF* when compared to *PO* and *TM.*
- Identification of the best performing strain; A natural *SC* strain (227) has been identified that produces melanin. It also grows better on *AF* when compared to 4.8 and 4.39.
- Confirmation that growth is possible at microgravity conditions; growth of *SC* may even be stimulated by microgravity.
- Confirmation that growth is possible at high radiation levels; *SC* is able to survive 200 Gy radiation for 3 days. This is not related to enhanced melanin formation.
- Small scale *AF/SC* biocomposite panels have been produced.
- Tools for extrusion of paste-like *SC* / substrate composites have been developed.
- Computational design strategies have been implemented for controlling robotic devices and for consequent deposition of mycelium based pastes.
- Different shaped 3D printed mycelium composite objects have been created.
- Growth on nanocellulose has been tested. Further experiments are needed to identify the most optimal match between the typology of nanocellulose and *SC* either or not in combination with *AF*.

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