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Development and Characterization of Radio-Photo-Autotrophic Biofilms: A Promising Candidate for Sustainable Life Support Systems

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ABSTRACT

Radiation exposure and oxygen deficiency represent major challenges in space exploration missions. This study explores the potential of engineered microbial biofilms as sustainable life support systems capable of providing radiation shielding and oxygen generation. Biofilms with radio-tolerant, photosynthetic, and nitrogen-fixing capabilities were developed using *Aspergillus niger* (A), *Bacillus subtilis* (B), and *Chlorella* sp. (C) in 96-well plates using modified yeast mannitol broth (YMB). Biofilm formation, biomass, and viability were assessed through microscopy and cell density mapping, crystal violet assay, and tetrazolium salt assay, respectively. To assess radiotolerance, UV-C radiation at an intensity of 3.4 W/m² was applied to developed biofilms under varying doses. After irradiation, biofilm integrity was assessed through microbial viability assays. The results revealed that AC and C biofilms produced significantly ($p < 0.05$) higher biomass compared to other biofilm formulations. The A, AC, and ABC biofilms amazingly maintained microbial viability after exposure to 6120 J/m² of UV-C radiation, a dose equivalent to that experienced by microbes during 30 minutes of direct exposure to the surface conditions on Mars. In conclusion, radio-photo-autotrophic algal–fungal–bacterial biofilms show promising potential as sustainable life support systems. Further research is needed to enhance the efficiency of these biofilms and explore their integration onto the surfaces of equipment or the skin of animals and humans, within the context of space exploration missions.

KEYWORDS: Biofilms, Life support systems, Radiation, Space exploration

INTRODUCTION

Radiation exposure poses significant challenges for humans as well as other biological systems. Harmful Radiation that comes through space or radioactive material has the potential to cause damage to biological life by creating reactive oxygen species (ROS) and inducing mutations in DNA. While Earth is protected from most of the harmful cosmic radiation due to its magnetic field and ozone layer, a certain amount of UV radiation does penetrate Earth's surface, also allowing certain terrestrial organisms to develop mechanisms to tolerate it.

Humans as a species that plan to be a multi-planetary species, there is a high probability of encountering high radiation environments including even those of ionizing capabilities. Thus, it is of vital importance to have proper radiation protection on biological material and equipment in such environments (Premarathna *et al*., 2024).

Before any type of extraterrestrial colonization, it also of great importance to have the basic necessities for life. Oxygen (O_2) is one such important necessity. Photobioreactors are studied to be used on such occasions as bioregenerative life support systems (BLSS) which would use up carbon dioxide (CO_2) and produce O_2 (Fahrion *et al*., 2021). However, radiation is a major challenge in systems where microorganisms rely on light, as they cannot be easily shielded from harmful parts of the spectrum.

For the problems stated above, efficient radiation shielding mechanisms would be the common answer. There are several existing methods to reduce such radiation levels which also have their own drawbacks. Thus, finding alternative ways for radiation shielding is important (Shunk *et al.*, 2020). As an example, Lead (Pb) is one of the commonly used elements to provide an effective shielding from ionizing radiation on certain occasions. However, Pb is also a heavy metal that possesses high toxicity, high weight, poor durability, and creates environmental disposal problems (Mortazavi *et al*., 2024). As an alternative approach, a biological radiation shield could be used in many instances with minimum side effects to the environment.

One such promising example is *Cladosporium sphaerospermum*, a fungus, tested aboard the International Space Station on its ability to attenuate ionizing radiation and it was found that a 1.7 mm thick melanized fungi layer reduced around 2.17% of ionizing radiation (Shunk *et al.*, 2020). This discovery highlights the potential of fungi to be used as a biological radiation shield.

Such fungi that are found in high radioactive environments, have developed

mechanisms to protect themselves from radiation-induced damages. They possess protective mechanisms like the production of secondary metabolites like melanin (Shunk *et al*., 2020). These fungi could be potential candidates for developing such a biological radiation shield. However, fungi cannot self-sustain due to their heterotrophic nature. One of the most important factors to consider in utilizing biological radio-tolerant microbes is a nutrient source, if they are to be used in radioactive environments where nutrients could be scarce and supplying a full nutrient package could be costly.

To overcome such limitations in biological radiation shields, it is crucial to establish a mechanism that enables continuous nutrient generation for the organisms involved. Here we suggest that, one way of achieving this is by utilizing a strong symbiotic relationship between nutrient generating organisms. The use of a photoautotrophic microorganism in the symbiotic system will produce carbohydrates and O_2 which could be used by the radio-tolerant fungi and the fungi itself could protect the algae in return. Furthermore, using a radio-trophic fungi could provide the biofilm with the ability to radiosynthesis where they convert gamma radiation energy to chemical energy using melanin, making it a potential candidate in building such biofilms (Shunk *et al.*, 2020).

This study focuses on developing a similar symbiotic system as a polymicrobial biofilm. In addition to the microorganisms, the biofilm matrix is composed of extracellular polymeric substances (EPS) secreted by the resident microorganisms. The EPS will have several advantages to the proposed system through providing resistance from environmental stress, acting as a carbon source for microbes, and trapping nutrients (Costa *et al*., 2018).

In the proposed system, *A. niger* will be used to provide radiotolerance to the system which is one of the species found in radiation-contaminated environments (Tibolla and Fischer, 2025). *Chlorella* sp. will be used as the photosynthetic organism and, in addition, a nitrogen-fixing bacterium, *B. subtilis*, will be used to maintain the sustainability of the biofilm by providing fixed nitrogen to the proposed system (Hashem *et al*.,2019).

The primary objective of this particular study is to engineer a tri-species biofilm capable of both photosynthesis and radiation tolerance as a sustainable life support system that promotes in-situ resource utilization (ISRU). This research serves as a foundational step towards the development of biofilm-based sustainable life support systems.

METHODOLOGY

Selection of Microorganisms

Aspergillus niger (A), Bacillus subtilis (B) and Chlorella sp. (C) were obtained from the NIFS culture collection as the radio-tolerant fungi, nitrogen fixing bacteria and the photoautotrophic algae, respectively. The microorganisms were revitalized in potato dextrose agar (PDA), nutrient agar (NA) and blue green-11 (BG-11), respectively, to be used in the polymicrobial biofilm formation.

Biofilm Formation in 96-well Plates

Ninety-six-well plates with a flat bottom were used in all experiments. Spore suspension of *A. niger* was prepared by flooding a fungal colony grown on PDA with a sterile solution of 0.9 % NaCl containing 0.025 % Tween 20, followed by filtration to obtain a fungal spore suspension (Cortesão *et al*., 2020).

B. subtilis cultured in Nutrient Agar (NA) and *Chlorella* sp. cultured in Bg-11 media were used as units of inoculums.

The growth media for biofilm development was prepared by combining yeast mannitol and Bg-11 broths. The media was added to 96-well plates in a manner whereas the total volume of the wells would be 200 µl after the inoculation of microorganisms. A volume of 30 µl of each microorganism was used as the unit of inoculum for A, B, C, AB, BC, AC, ABC biofilm combinations. The plates were then incubated in room temperature and light conditions for 1 week to allow biofilm formation under static conditions.

Biofilm Confirmation

For proper visualization and confirmation of biofilm structures, biofilms were stained with Lactophenol cotton blue, and visualized under a light microscope.

For further visualization and confirmation of biofilm structures in the microscopic images, the colour contrast between the green algae, the blue-stained fungal hyphae and bacteria were used. Digital microscopic images were processed using Python and density maps of blue colour and green colour distribution were generated separately to infer the spread of organisms.

Crystal Violet Assay to Compare Biomasses of the Developed Biofilms

After the incubation period, planktonic cells and remaining media were washed out from the wells, allowing only tightly adhered biofilms to remain in the wells (Chojniak *et al*., 2017; Wilson *et al*., 2017). The wells were then stained by adding 0.1% crystal violet (CV). After incubating for 10 minutes, the excess dye

was removed from the wells by washing using Phosphate buffer saline (PBS). Dye embedded into the biofilm biomass was extracted using 95% ethanol, and optical density values of the extractant were measured at 620 nm using a 96-well plate reader. According to the Beer–Lambert law, absorbance values are proportional to biomass concentration, supporting the hypothesis that higher absorbance indicates greater microbial growth.

Development of a UV-C Irradiation Setup

UV-C treatment was given inside a class II biosafety cabinet using a T6, 30 W UV-C germicidal lamp at 254 nm. A flux meter was used to measure the UV-C intensity at several positions of the biosafety cabinet.

A stage was prepared inside the biosafety cabinet up to a position which received an UV-C intensity of 3.4 W/m^2. This intensity was chosen to provide UV-C conditions close to those on the Martian surface (Cockell and Andrady, 1999).

UV-C Radiation Treatment

Selected combinations of biofilms were developed in 96-well plates as explained above. Spent growth media and planktonic cells were removed from the wells. The remaining biofilms were directly exposed to an UV-C intensity of 3.4 W/m^2 with varying time periods to change the received radiation dosages of 100 Jm^2, 1000Jm^2, 3060Jm^2, and 6120 Jm^{-2} (Onofri *et al*., 2008). UV-C dose was calculated according to the following equation (Cortesão *et al*., 2020).

$$t\,(s) = \frac{D\,(J/m^2)}{I\,(W/m^2)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

where t = time (seconds); D = desired radiation dose (J/m^2), I = UV-C intensity (W/m^2) measured by a flux meter.

Biofilm Viability after UV-C Irradiation

Irradiated samples were incubated with 0.5% 2,3,5-Triphenyltetrazolium chloride (TTC) solution for three hours. Biofilm viability was confirmed by using the colour change, due to the reduction of TTC into red colour formazan (Wilson *et al*., 2017). The samples were visualized using the light microscope to monitor biofilm structures after irradiation. The irradiated biofilm samples were also inoculated in NA for testing their viability.

Statistical Analysis

Digital image processing was done using Python scripts, executed in Google Colab. Minitab version 20.4 was used to perform statistical analysis including ANOVA and Tukey's HSD test.

RESULTS AND DISCUSSION

Biofilm Development

Microscopic imaging revealed that the algae and bacteria have clearly colonized the fungal mycelium, forming a well-structured algal-fungal-bacterial biofilm (Figure 1). The overlapping nature of density maps also confirmed that the *Chlorella* sp. and *B. subtilis* have well colonized the *A. niger* mycelium.

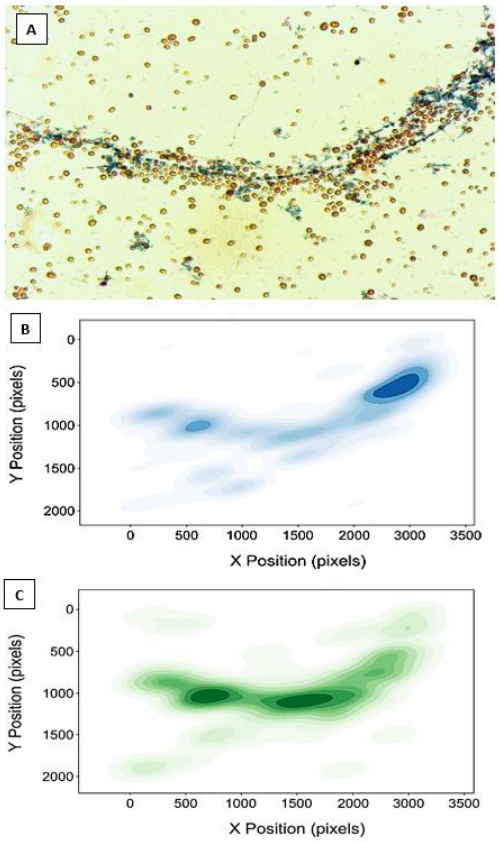

Figure 1. (A) Microscopic view of *Aspergillus niger-Bacillus subtilis-Chlorella* sp. biofilm at 100x magnification, (B) Processed image showing the map of *A. niger* and *B. subtilis* cells densities, and (C) Processed image showing the map of *Chlorella* sp. cell density, with respect to their position in pixels

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Biomasses of Developed Biofilms

Resident microbes in the developed biofilms and their extracellular polymeric substance (EPS) are generally stained by Crystal violet giving a measure of how much biofilm amount is formed within a given time. The AC and C biofilms yielded a significantly ($p < 0.05$) higher biomass at one week after inoculation (Figure 2). This suggests a strong synergistic interaction between *A. niger* and *Chlorella* sp., which appears to be disrupted by the addition of *B. subtilis* to the system (Xu *et*

al., 2025). The observed reduction in biomass in the ABC biofilm may be due to an inhibitory effect of *B. subtilis* on *A. niger*. However, further investigation is required to determine the exact cause of this reduction. Optimization strategies are also necessary to enhance the performance of the ABC biofilm, as it remains the primary target for development.

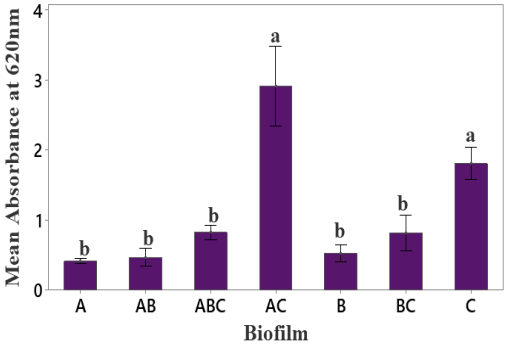

Figure 2. Comparison of biomasses of the developed biofilms by crystal violet assay. According to the Beer–Lambert law, absorbance values are proportional to biomass concentration, supporting the hypothesis that higher absorbance indicates greater microbial growth *A: Aspergillus niger, B: Bacillus subtilis, C: Chlorella sp., AB: A. niger-B. subtilis, AC: A. niger-Chlorella sp., ABC: A. niger-B. subtilis-Chlorella sp. Individual standard errors are used to calculate the intervals.*

Viability of Developed Biofilms after UV-C Irradiation

The A, AC, and ABC biofilms remained viable after exposure to 6120 J/m² of UV-C radiation, a dose equivalent to that experienced by microbes during 30 minutes of direct exposure to the harsh surface conditions on Mars (Table 1). However, their viability level on the different doses needs to be quantified using an optimized protocol.

Table 1. Assessment of Biofilm viability under TTC assay after UV-C irradiation

Biofilm	UV-C Dosage (Jm^2)			
	100	1000	3060	6120
A	+	+	+	+
AC	+	+	+	+
ABC	+	+	+	+

A: Aspergillus niger, AC: Aspergillus niger–Chlorella sp., ABC: Aspergillus niger–Bacillus subtilis–Chlorella sp. +: viable under TTC.

Microscopic images taken after irradiation and post incubation with TTC show that the biofilm structure of ABC was still viable (red colour) and intact even after a UV-C dose of 6120 Jm^{-2} (Figure 3A). Viability was further confirmed by the growth of the irradiated biofilms in NA plates where irradiated biofilm ABC showed considerable colony growth (Figure 3B). However, the percentage of survival of individual species in the biofilm was not quantified at this level.

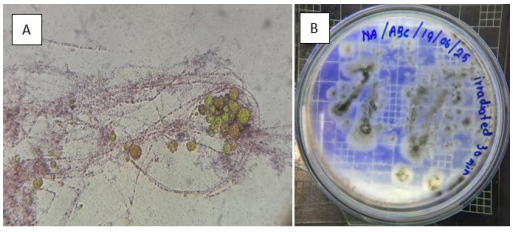

Figure 3. A: Light microscopic image of *ABC* biofilm after incubation with TTC, B: Irradiated biofilm structure of *ABC* grown in NA *All images are taken from biofilms subjected to 6120 Jm^{-2} as the highest dose tested.*

Furthermore, optimization of the ABC biofilm is necessary to enhance biomass production, as it represents the targeted system with the unique advantage of self-sustained nitrogen fixation, a feature absent in the AC combination. Adjustments to environmental parameters are required to support optimal growth and stability of the ABC consortium. In addition, the biofilm should be evaluated for its tolerance to a broad spectrum of ionizing and non-ionizing radiation, as well as its capacity to produce oxygen under simulated space-like conditions.

CONCLUSIONS

This study lays the groundwork for regenerative life support systems by engineering a photosynthetic, radiation-tolerant algal–fungal–bacterial biofilm. Future research should focus on optimizing these biofilms, testing their resilience under combined space-like stressors, and exploring their integration onto equipment or even biological surfaces for enhanced functionality.

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