Master Thesis

Living Labels

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July 21st, 2023

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Master Thesis
MSc. Integrated Product Design

21-07-2023
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Executive Summary

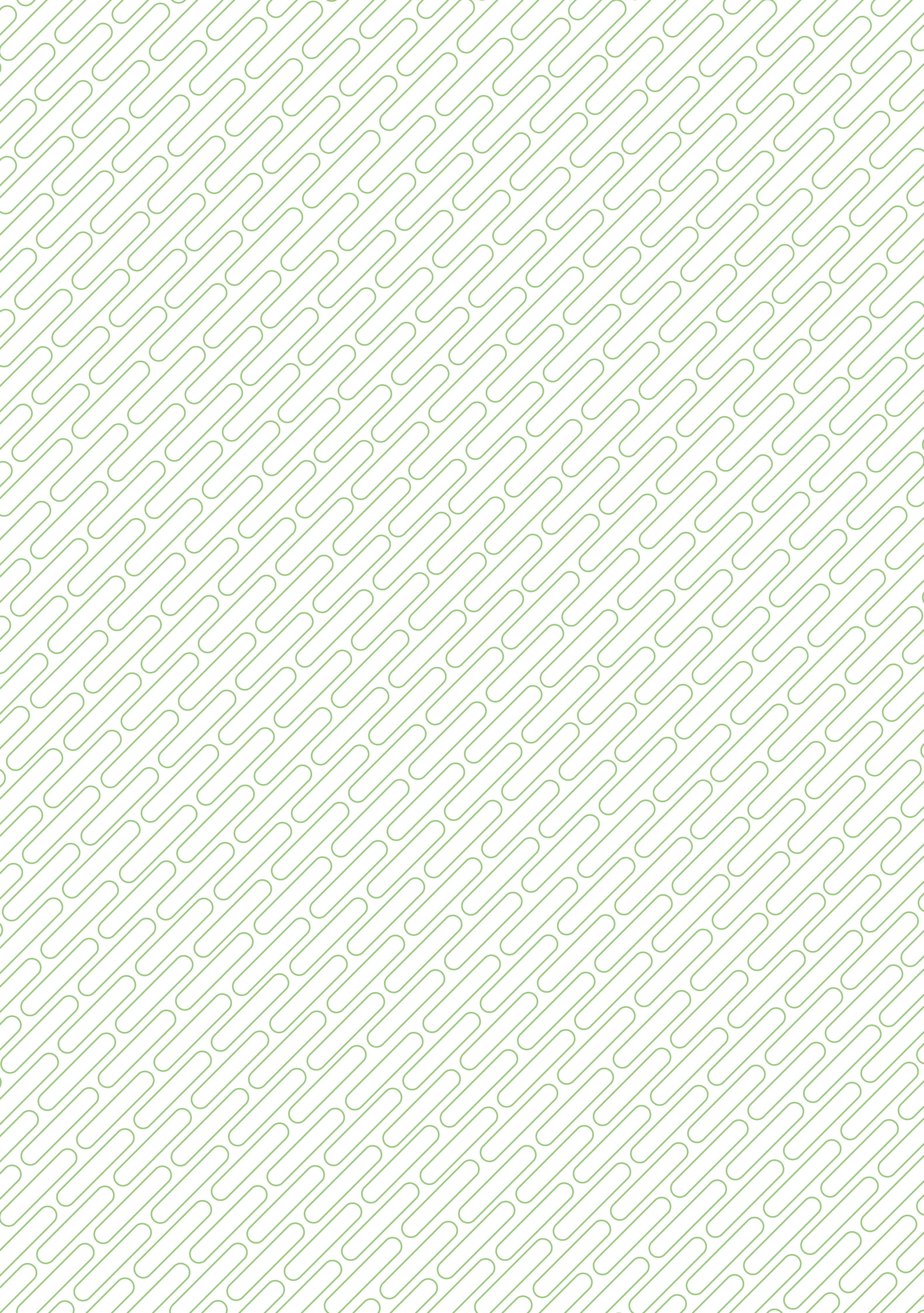
Flavobacteria are marine organisms that produce vivid, angle-dependent colour as their cells organise into structures that interact with light. These optical structures, resulting in structural colour, are influenced by environmental factors such as temperature and humidity. In this graduation project, the aim was to integrate living Flavobacteria into a flexible, sustainable material, creating labels that communicate environmental signals through changes in the structural colour of the embedded Flavobacteria. Understanding how to maintain Flavobacteria and their structural colour, outside the laboratory can be the steppingstone to make the entry of these microorganisms into the world of design.

The initial phase of the project focused on understanding the requirements for Flavobacteria to make structural colour outside the controlled laboratory environment. This understanding was crucial to design in a way that accommodates the unique characteristics of these microorganisms. Temperature was selected as the primary environmental stimulus to address, given its significant impact on the size and colour of the Flavobacteria colony. Additionally, temperature changes can be transferred through materials and therefore enable a sealed label without the necessity of openings to let environmental stimuli through.

The second phase involved an iterative experimentation process, which aimed to identify a suitable top material of the label that would facilitate the formation of structural colour by Flavobacteria. Additionally, efforts were directed towards finding an appropriate growth medium for Flavobacteria within the living label and devising an activation method that would initiate their growth exclusively during the product's use phase. The outcomes of this iterative process were then integrated into a vision for the living label.

The project continues by presenting a future vision for the living label and proposing additional guidelines for designing with Flavobacteria. This vision draws upon literature research as well as the findings and insights gained through this graduation project. Further research is recommended regarding the characterization of the temperature influence on Flavobacteria, improving the surface texture of the medium used in the living label

To conclude, by combining existing knowledge with the project's outcomes, a first proof of concept for a living label is proposed, paving the way for future exploration and application of these living microorganisms in innovative design contexts.



Preface

Sustainability is an increasingly important topic in today's world. However, we are bombarded with terms like "bio" and "recycled", making it difficult to navigate through greenwashing and marketing tricks. While the focus on sustainability is commendable, it often feels like the proposed solutions are too small to address the larger problem at hand.

As a product designer, I often find myself in a difficult position, since the most sustainable way of being a product designer would be to design no product at all. To still find a direction throughout my academic career, I have tried to understand the motivations behind our way of product usage. Therefore, in many of my previous projects I have focused on how to bring meaning into products so that we as society will consider them as more valuable, emphasizing the need to move away from non-circular consumption patterns. Yet, these efforts as well seem insufficient to make a substantial impact.

Biodesign is the first direction that has given me hope since it suggests that the future doesn't have to be just a slightly less detrimental version of the present; it can be even better than we imagine. By collaborating with nature instead of exploiting or extracting from it, we can foster a culture that respects and harnesses the multitude of benefits nature has to offer. Nature is the most experienced engineer out there. There are solutions all around us, all we need to do is look for them.

Glossary

Agar
Gelling agent extracted from seaweed, used to create hydrogels and solidify microorganisms' growing media

Demi water
Abbreviation for demineralized water, which has all minerals, salts and ions removed

Inoculate
Placing a microorganism onto a surface

Iridescence
Angle-dependent colour

Petri dish
Shallow, transparent dish to hold growth medium

Structural Colour
Colour produced by light interacting with micro- or nano-structures

Viability
The ability for bacteria to live successfully

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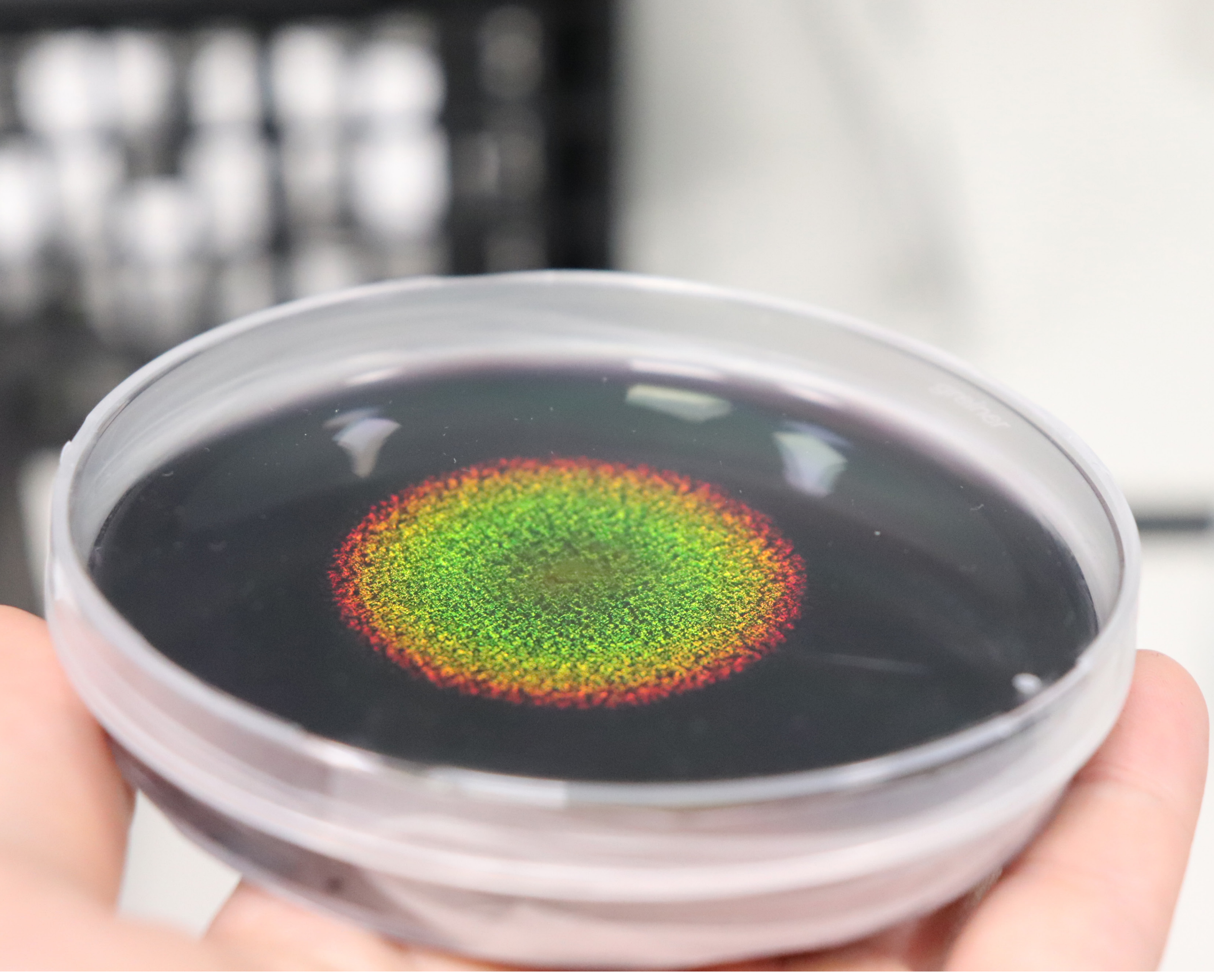

CH. 1

Project Overview

Introduction

At the start of this graduation project, a clear aim and scope were formulated to frame the project. Additionally an approach was set up for the design process that will be followed in the coming chapters. This chapter outlines the specifics of the set up of this project.

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Method

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1.1 Introduction

Within the field of biodesign, designers, artists and scientists are collaborating with living organisms to produce new materials with ecological benefits. As society's focus on sustainability intensifies, there is a growing interest in design solutions that prioritise the well-being of the planet. Moreover, design with living artefacts introduces novel possibilities for interaction based on the unique traits of the organisms involved.

Various organisms are being explored for biodesign purposes due to their distinctive characteristics. For example, bioluminescent algae offer possibilities for light emission (Barati et al., 2021), while algae are utilised for air, soil, or water purification (Ansar et al., 2022). In this graduation project the emphasis is on marine Flavobacteria. Flavobacteria possess the ability to produce vibrant, angle-dependent colours as their cells organise into structures that interact with light (Figure 1.1). The Flavobacteria colony is subject to change over time and in response to environmental factors such as temperature and humidity (Groutars and Risseeuw et al., 2022).

To date, the observation of cell organisation in Flavobacteria, which results in their structural colour, has been limited to laboratory settings. This poses a challenge when attempting to apply this phenomenon to the design of everyday objects. However, envisioning the potential of Flavobacteria's structural coloration outside the laboratory enables intriguing possibilities, such as integrating it into everyday objects like, for instance, a living sensor. In this context, a living sensor capable of communicating environmental changes through vivid colorations could potentially replace conventional electronic sensors.

This project aimed to embed Flavobacteria within labels that can function as standalone sensors, conveying information about temperature and humidity changes to users. These labels can also be attached to products that are sensitive to environmental variations and the passage of time, such as perishable goods and plants. By doing so, users gain a better understanding of the needs and conditions associated with these products.

This graduation project was carried out in collaboration with Hoekmine and Bio4Life under the KIEM project Living Circular Labels. Bio4Life specialises in the production of biodegradable labels (Bio4life - Supplier of Biodegradable Labels, 2022). Hoekmine, a company working with Flavobacteria, offers microbiological expertise throughout the course of this project.
In collaboration with designers, they have been researching the potential of Flavobacteria for producing sustainable colourants for application in everyday products (Hoekmine | Hoekmine BV, n.d.). They also envision biosensors, which would minimise the use of increasingly demanded electronic sensors, and thus, the implementation of scarce and toxic materials that often remain improperly handled at the end of their lifecycle (Nationaal (W)EEE Register, 2021).

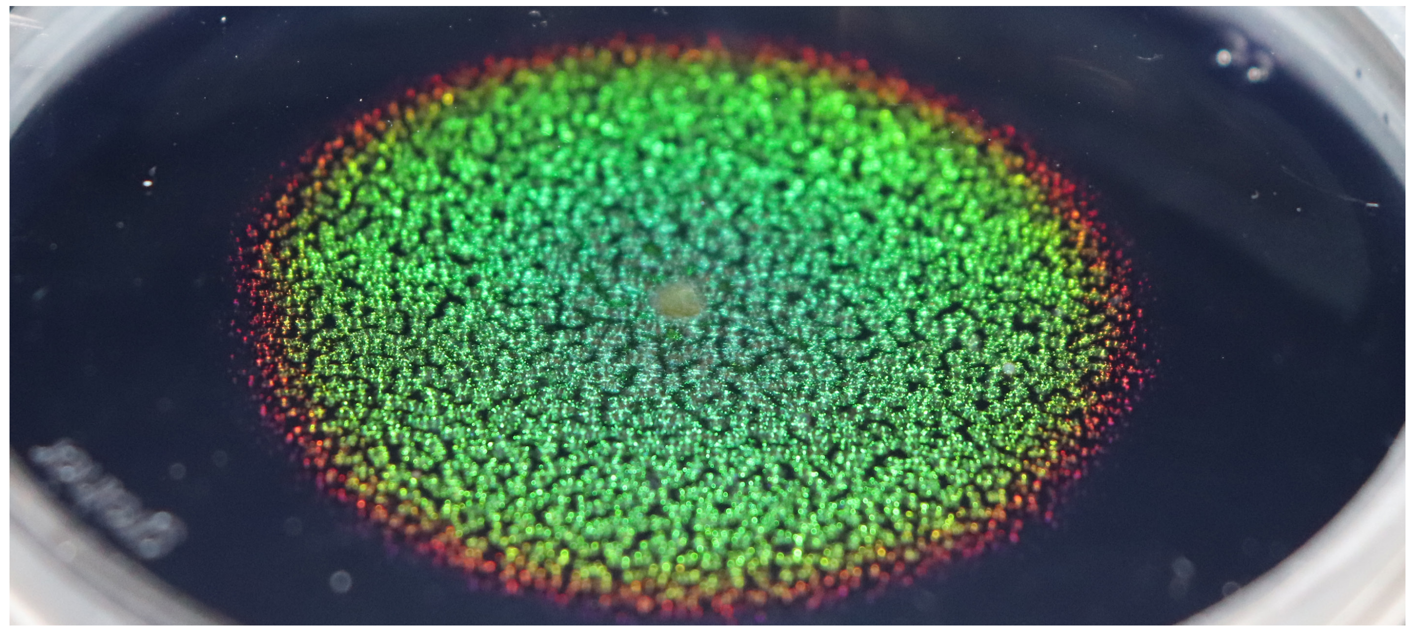

Figure 1.1 *- Cellulophaga lytica, grown on MAR medium for 4 days under standard conditions*

1.2 Aim & Scope

1.2.1 Aim

The aim of this project is the integration of living Flavobacteria into a flexible, sustainable material, creating labels that communicate environmental signals through changes in their structural colour.

Secondly, 'activation' methods of Flavobacteria growth in the living label will be explored to avoid Flavobacteria's growth until its activation by the users. By doing this, this project aims to bridge the gap between microbiology and embodiment design and contribute to the development of a circular economy where technology and organic systems merge in the design of living circular labels.The living label is designed with the application of a label for perishable food products in mind.

1.2.2 Scope

In this project, an interdisciplinary approach is followed touching upon growing design, microbiology and materials science. The relevance of these disciplines to this graduation project is explained below.

Growing Design
Growing Design is defined by Camera & Karana (2017) as the fabrication of materials and products from living organisms. It entails working in an interdisciplinary context and finding sustainable solutions for product design (Camere & Karana, 2018). This perfectly outlines the core of this project, that is at the intersection between biology and design. Furthermore, the development of a design with integrated Flavobacteria, will result in a stepping stone towards a sustainable alternative to electronic sensors.

Microbiology
In order to understand how to design with and for Flavobacteria, it is important to understand the microorganism and its needs. This project includes extensive literature research on Flavobacteria and laboratory experiments that will extend our knowledge on their behaviour. Furthermore the chair of this project, Dr. J. Martins, is an expert on microbiology and can therefore provide support in this field. Additionally, the microbiologists from Hoekmine are also part of the project consortium.

Materials Science
Apart from the microorganism, this project is also about materials science as a large part of the design process consisted of experimenting with different materials to observe the behaviour of Flavobacteria when combined with them. Additionally, different (combinations of) materials were fabricated to accommodate the needs of the studied bacteria.

1.3 Approach

Extensive literature research was conducted from the project brief onwards to gain an understanding of the specific needs and behaviour of Flavobacteria. This research aimed to establish a benchmark of the general existing knowledge about these microorganisms. Furthermore, the sensing capabilities of Flavobacteria were validated, and in combination with the literature research were utilised to define the design requirements.

With the design requirements in place, the iteration process was started by defining three distinct components of the label: the top layer, the Flavobacteria and their growth medium and the bottom layer. This categorization was based on envisioning the living label in its simplest form, as depicted in figure 1.2.

Based on the three identified components of the label (top layer, Flavobacteria and growth medium and the bottom layer), three distinct directions were established for experimentation. These directions included the exploration of biodegradable and transparent options for the top layer of the label, the development of a suitable medium for Flavobacteria's growth, and the investigation of different activation methods for Flavobacteria growth as well. The bottom layer did not necessitate a separate iteration process since it is not in direct contact with the Flavobacteria and therefore is a less challenging part of the living label. Each direction was subjected to an iterative experimentation process.

Following the iterative experimentation phase, the results obtained from each direction were integrated into a design that addressed the specific requirements of the living label. The design was then partially validated to ensure its feasibility and effectiveness.

An additional consideration in the design process was the need to keep Flavobacteria viable throughout the project to ensure young and active cells for the experiments.

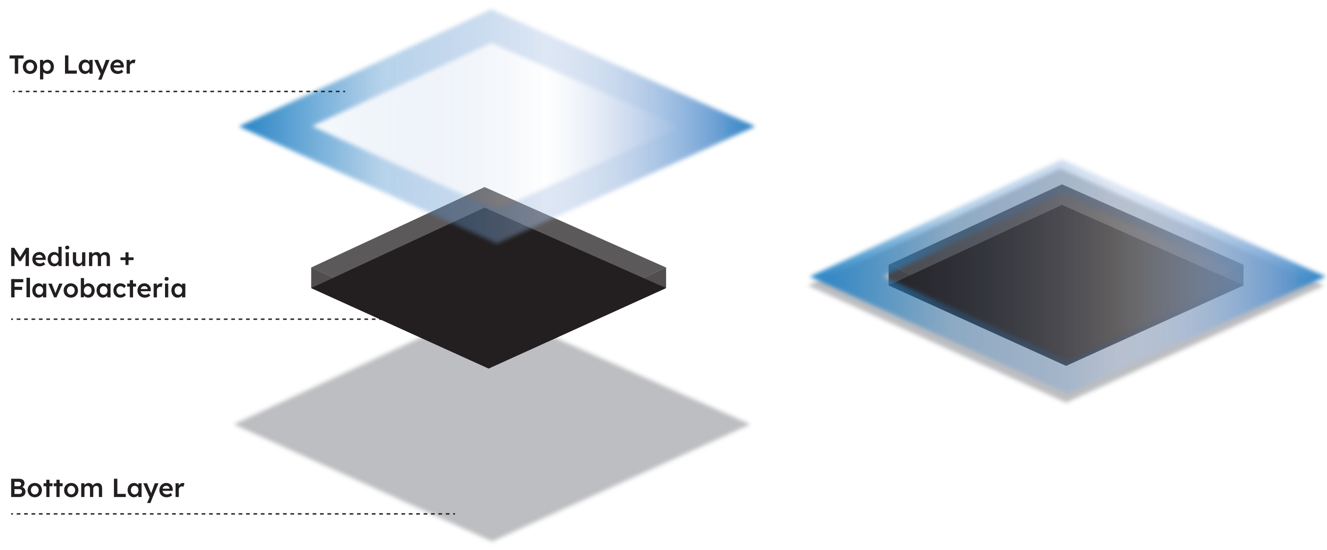

Figure 1.2 *- Living label exploded view*

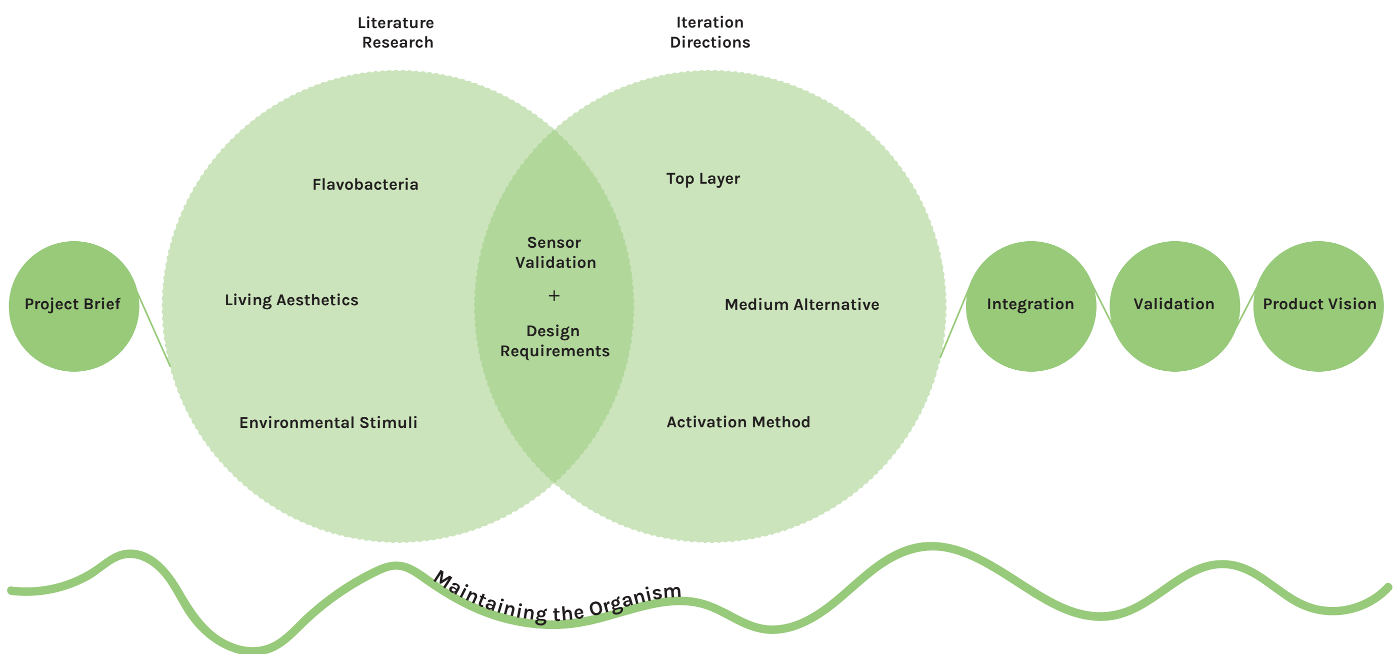

Figure 1.3 *- Design Process*

Cover Image - *Design to Fade by Puma x Living Colour - Picture retrieved from (Ilfa, 2020)*

CH. 2

Background Information

Introduction

After having framed the project, background information on biodesign and Flavobacteria was neccesary to gain a better understanding of the design problem. In this chapter, a summary of the literature research on these topics is presented.

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Method

Literature research

2.1 Biodesign

For centuries, designers have sought inspiration from nature, resulting in the emergence of bioinspired innovation. This encompasses various approaches, such as biomimicry, which involves replicating nature's engineering principles; biomorphism, which draws aesthetic inspiration from natural forms; and bioutilization, which involves utilising biological materials in designs. Additionally, the concept of biophilia is commonly applied in the built environment to enhance the connection between humans and nature. However, a common limitation among these approaches is that although they draw inspiration from nature, the materials used in the designs are typically extracted from nature and often lack a circular approach.

Biodesign, on the other hand, represents a significant step towards a future where we collaborate with living materials to create more environmentally sustainable artefacts. Two noteworthy examples of biodesign projects are "Indus" and "Design to Fade", both of which harness the unique characteristics of microorganisms to create innovative designs.

Indus by Bio-integrated Design Lab of UCL
Indus is a wall system designed to clean wastewater through bioremediation by microalgae (Figure 2.1). It is designed with rural communities in mind to regenerate water for reuse. Water is guided over vein-like channels, filled with immobilised microalgae cells, that contribute to optimise heavy metal uptake (Ucl, 2020).

Design to Fade by PUMA x Living colour
Living colour by Laura Luchtman and Ilfa Siebenaar is an example of a biodesign project exploring the possibilities of natural textile dyeing with bacteria that produce pigment (Figure 2.2) (Ilfa, 2020b). With this project the designers show an alternative to the toxic clothing dyes, through the use of bacteria to create a biodegradable dye. This approach results in little water consumption and low dyeing temperatures without the use of any toxic chemicals or textile treatments. The applied bacteria occur naturally and feed on nutrients that are fermented into pigments. These bacteria are cultivated on the textile while also dyeing it (Ilfa, 2020b). The designers teamed up with Puma for the collection 'Design to Fade' Contributing to the very first bacterial dyed sportswear collection (Ilfa, 2020).

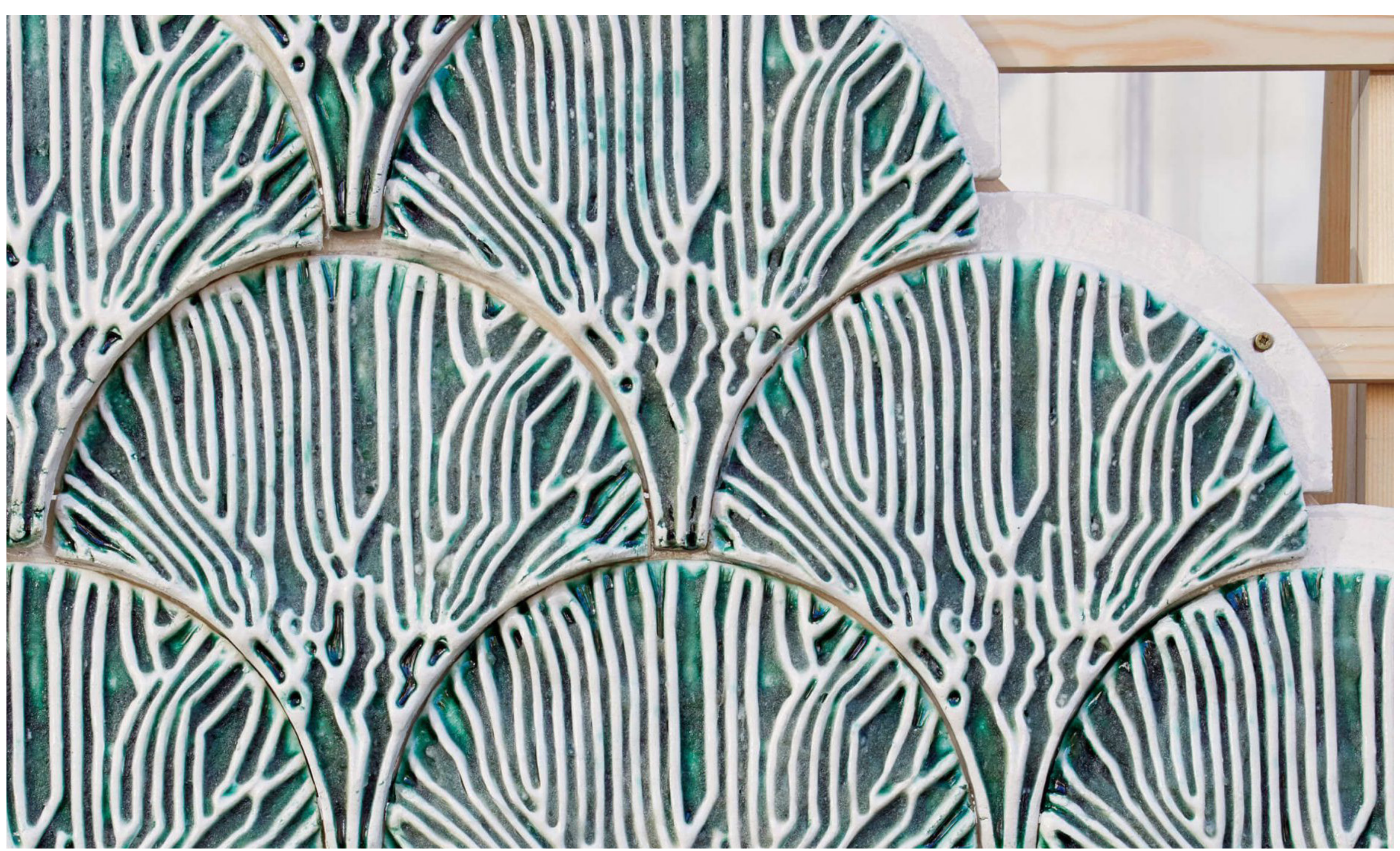


Figure 2.1 *- Indus by Bio-Integrated Design Lab of UCL. Picture retrieved from (Aldeghi, 2022)*

Figure 2.2 - *Design to Fade by Puma x Living Colour - Picture retrieved from (Ilfa, 2020)*

2.1.1 Livingness

The design potential of the example "Indus", lies in the use of living organisms, however not every Biodesign example uses living organisms in the use phase of the product. In the field of Biodesign, a distinction can be made between living and non-living artefacts. Non-living artefacts integrate living organisms into design primarily as a material source while living artefacts harness the potential of living organisms in the use phase.

An example of a non-living artefact is for instance the previously mentioned "Design to Fade" collaboration from Puma and Living Colour, where the dye that is produced by bacteria is used to dye clothing, but the bacteria themselves are not alive in the use phase. Another examples of non-living artefacts are the wall panels from Mogu (Figure 2.3), that use mycelium as a material source, but the organism is not alive in the use phase. This is because the mycelium is made inert before the use phase by slow drying (Muvobit, 2022).

Artefacts that are living, expand the livingness to the use phase (Karana et al., 2020). As described in the paper by Karana et al. (2020), livingness can be integrated in a design by prolonging it to the use time of artefacts so that the design outcome is more than just a sustainable material alternative. This creates new design possibilities. A smart material can be made with no need for the supply of external energy and mechanical or electrical control by relying on the responsive capacity of the natural systems (Karana et al., 2020). In the field of Biodesign, there are still many living organisms to be explored and characteristics to be researched for future applications. In this project the focus is on Flavobacteria.

Figure 2.3 *- MOGU, picture retrieved from (Muvobit, 2022)*

2.2 Flavobacteria

Flavobacteria are non-spore forming, rod-shaped bacteria that exist in various habitats like freshwater, marine and soil environments (Bernardet & Nakagawa, 2006;Pati et al., 2011). The length of Flavobacteria cells typically ranges from 1.5 to 10 µm, as can be seen in Figure 2.4, while their width falls within the range of 0.3 to 0.4 µm (Pati et al., 2011). While the majority of Flavobacteria are non-pathogenic, certain strains can act as pathogens, causing diseases in plants, fish, and even humans (Waśkiewicz & Irzykowska, 2014).

Regarding their motility, Flavobacteria employ a gliding mechanism when moving on semi-solid surfaces (Bernardet & Nakagawa, 2006). However, it is important to note that gliding motility cannot occur when Flavobacteria cells are suspended in a liquid medium (Bernardet & Nakagawa, 2006; Bernardet et al., 2002).

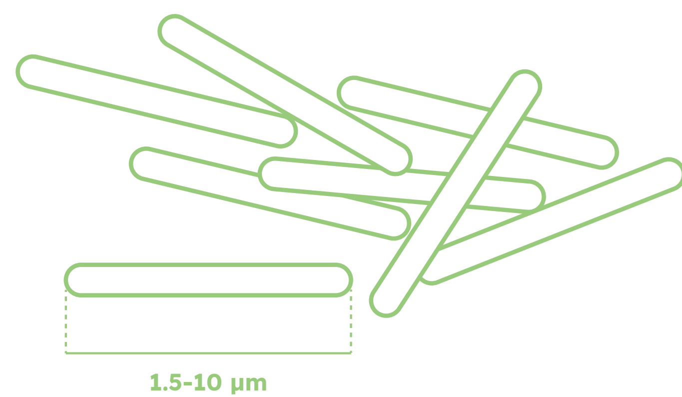


Figure 2.4 *- Rod-shaped Flavobacteria*

2.2.1 *Cellulophaga lytica*

In this project, the specie of Flavobacteria used is *Cellulophaga lytica* (abbreviated henceforth to *C. lytica*). The used strain, PLY A2, was isolated from Plymouth. In the instance that Flavobacteria are discussed in this report, this specific strain of *C. lytica* is referred to. In Figure 2.5 the classification of *C. lytica* can be seen.

C. lytica, as reported by Pati et al. (2011) and Kientz et al. (2012), is an aerobic bacterium, thriving in the presence of oxygen, and also halophilic, meaning it prefers saline environments. *C. lytica* grows optimally within a temperature range of 20°C to 25°C (Kientz et al., 2012)

When suspended in a liquid medium, the bacterium exhibits a yellow colour, attributed to the production of zeaxanthin pigment (Pati et al., 2011; Prabhu et al., 2013). Notably, when cultivated on a semi-solid surface, *C. lytica* demonstrates the ability to generate structural colour (Figure 2.6).

Domain	Bacteria
Phylum	Bacteroidetes
Class	Flavobacteriia
Order	Flavobacteriales
Family	Flavobacteriaceae
Genus	Cellulophaga
Species	*Cellulophaga lytica*
Strain	*C. lytica PLY A2*

Figure 2.5 *- Classification of C. lytica*

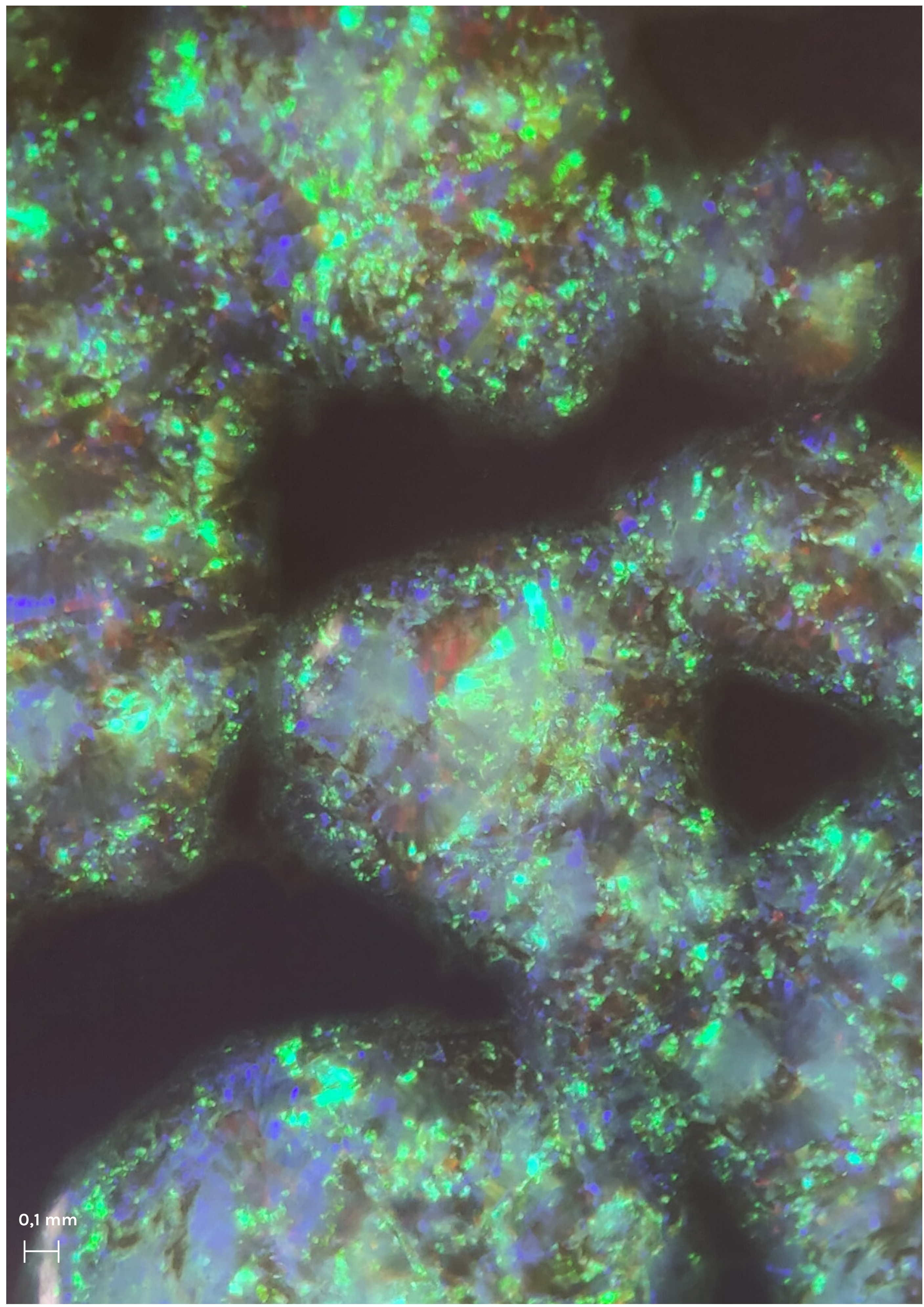


***Figure 2.6 -** Cellulophaga lytica, 50x magnification under microscope*

2.2.2 Structural Colour

Flavobacteria grown on a semi-solid surface, can show angle dependent structural colour (Groutars and Risseeuw et al., 2022). This angle dependent colour, alternatively referred to as iridescence, results from the structural colour that is formed by the organisation of the cell population in the colony biofilm (Kientz et al., 2016). Figure 2.7 shows Flavobacteria's cell organisation.

In nature, there are several types of phenomena that result in the colours that can be perceived. These encompass bioluminescence, pigments and structural colours. Bioluminescence emerges as a consequence of a chemical reaction, pigments derive their colouration through selective absorption of light, whereas structural colours arise from selective reflection of light (Tong et al.,2013). Structural colouration is achieved through the interaction of micro- or nanostructures with light. The structural colour that can be perceived is often the result of completely transparent materials (Parker, 2000).

In the case of *C. lytica*, the cells organise into a 2D photonic crystal (Hoekmine BV, n.d.). This intricate structure consists of multiple layers, which, when interacting with specific wavelengths of light, creates detectable colour through positive interference (Hoekmine BV, n.d.), as can be seen in figure 2.8.

While the prevailing structural colour observed in a *C. lytica* colony appears green with red edges, the colour depends on the conditions. As illustrated in figure 2.9, the green appearance of the colony originates from the interaction of the structure with wavelengths of visible light. Red is seen at the edges of the colony because the cell density is lower there, resulting in a slightly different light interaction.

Figure 2.7 - *Cell organisation C. lytica picture retrieved from (Hoekmine BV, n.d.).*

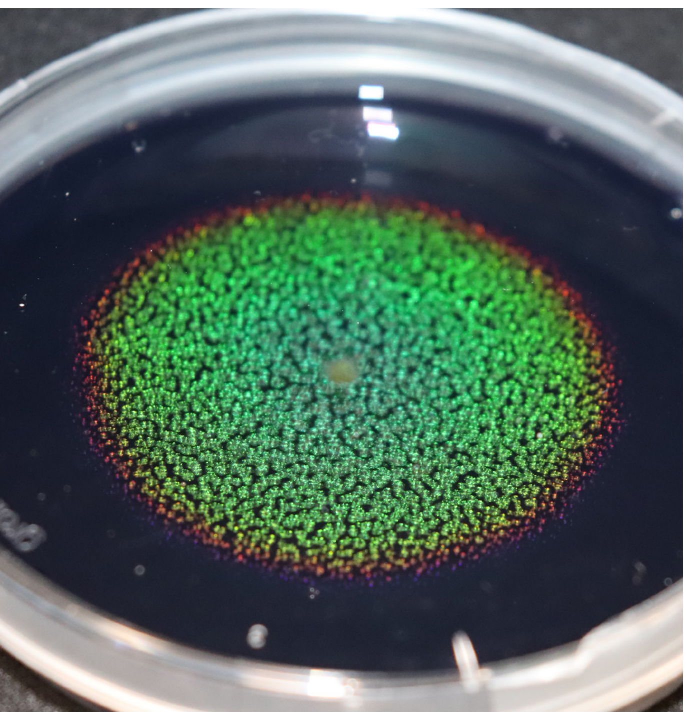


Figure 2.8 - *Cellulophaga lytica, grown on MAR medium for 4 days*

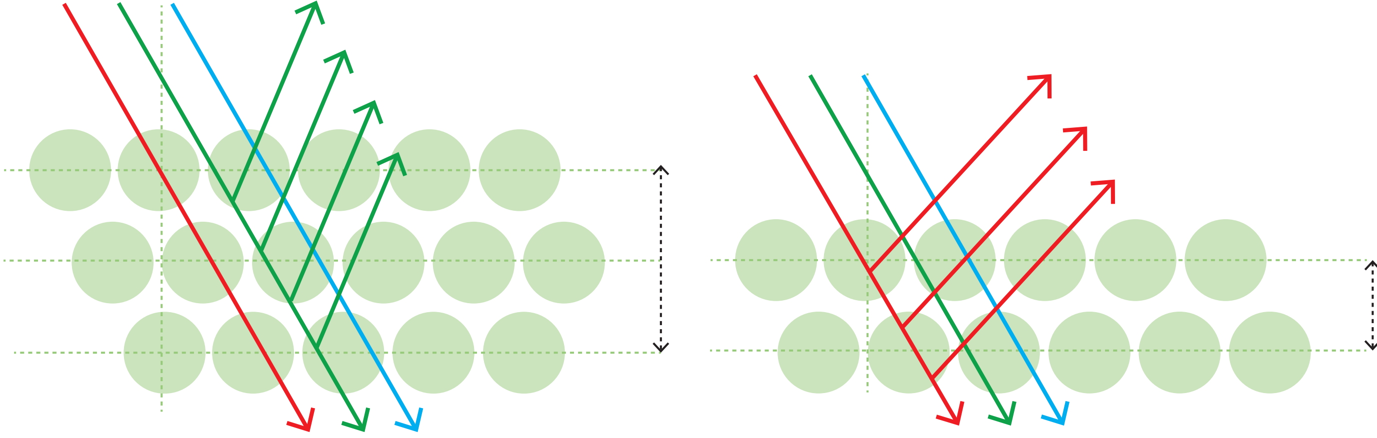


Figure 2.9 - *2D photonic chrystal in Flavobacteria colony*

Iridescence

The phenomenon of iridescence in *Cellulophaga lytica* results from the structural colour. Iridescence refers to the property wherein the perceived colour undergoes changes based on the viewing angle and orientation. The organisation of *C. lytica's* cells, their age and their microbial density significantly impacts the appearance of the iridescence (Kientz et al., 2012;Groutars and Risseeuw et al., 2022). A summary of these effects is represented in the figure 2.10.

The phenomenon of iridescence is demonstrated in Figure 2.11a and b, in which a Flavobacteria colony cultivated in a fridge at a temperature of 4°C was photographed from various angles. Given that the perceived colour is angle-dependent, capturing accurate representations can be challenging, particularly when comparing different colonies. Previous studies have indicated that Flavobacteria exhibit the most striking visual effect when viewed from an angle of approximately 45° (Groutars and Risseeuw et al., 2022). Therefore, in this project, the samples were photographed from this specific angle. Additionally, a black background was used to create a stark contrast with the structural colour of the Flavobacteria, thereby enhancing their visibility. To achieve this, nigrosine was incorporated into the growth medium of the Flavobacteria, as outlined in the paper by Groutars and Risseeuw et al. (2022).

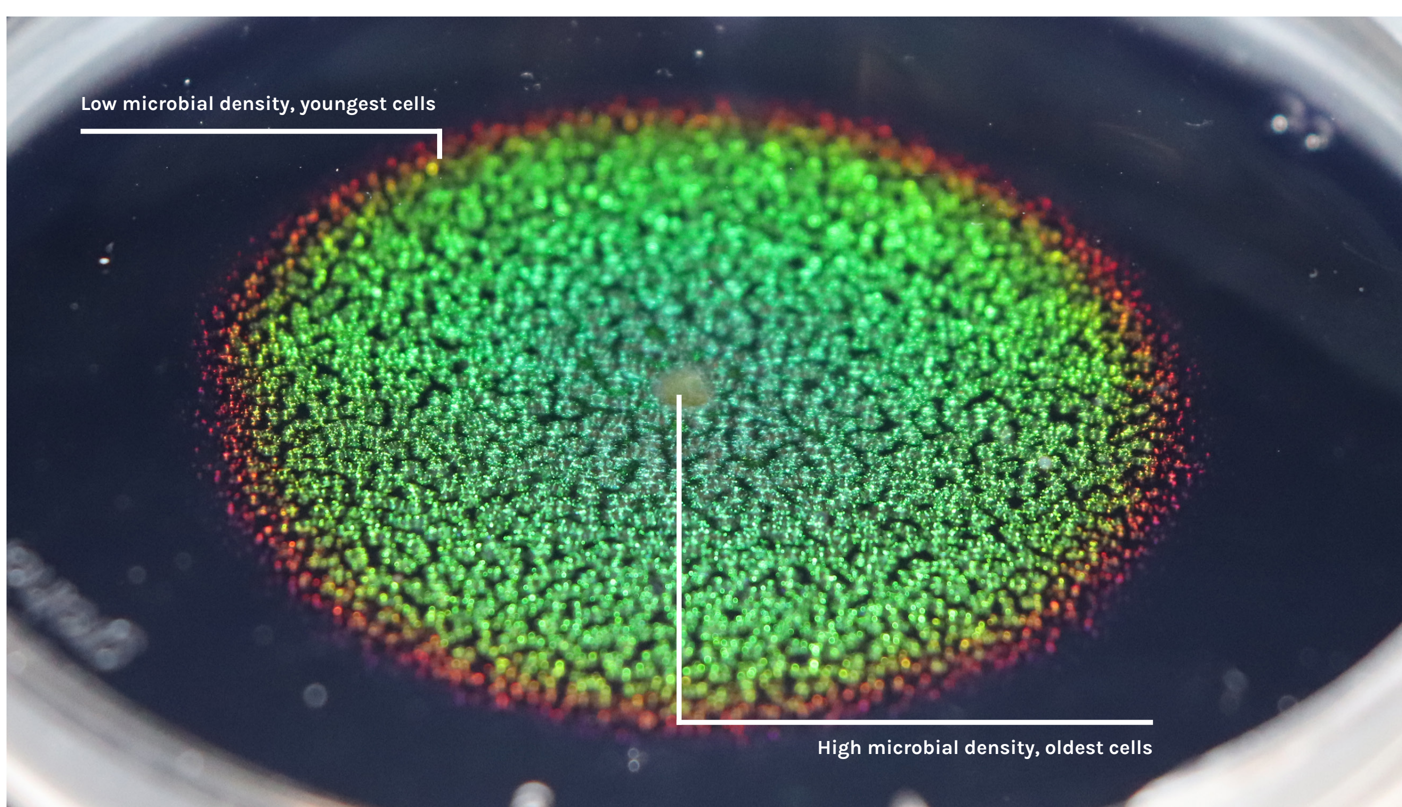


Figure 2.10 - *Cellulophaga lytica, grown on MAR medium for 4 days, standard conditions, captured at approximately 45°*

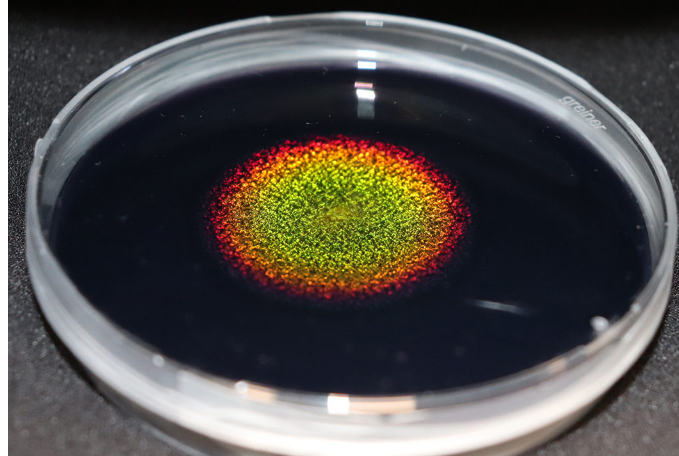


Figure 2.11a - *Cellulophaga lytica colony, 4 °C, MAR medium, 30 days, viewing angle of approximately 30°*

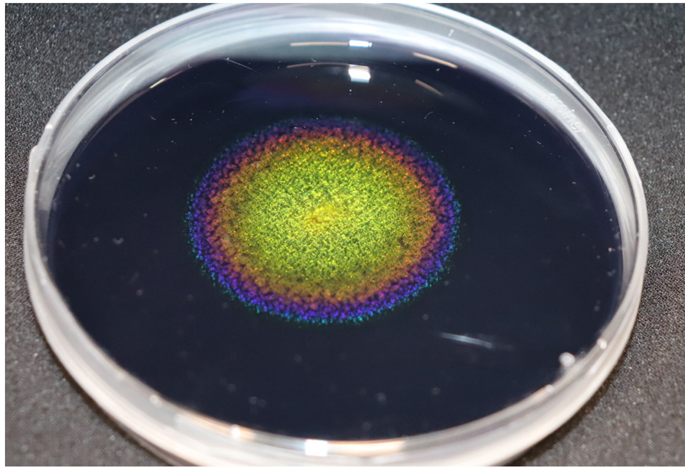


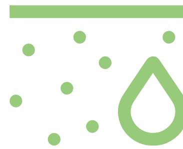
Figure 2.11b - *Cellulophaga lytica colony, 4 °C, MAR medium, 30 days, viewing angle of approximately 60°*

2.2.3 Requirements for Growth and Structural Colour

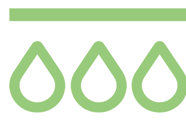
Requirements

There are several requirements needed for Flavobacteria to grow and be able to make structural colour. These are listed below.

Oxygen
Flavobacteria are aerobic, so first and foremost they need oxygen.



Salinity
The presence of essential seawater components is required for iridescence.



Humdity / Hydrogel
Humidity is needed for growth and a semi-solid, hydrated surface is needed to show structural colour. This surface is often created with agar as a gelling agent. The optimum agar concentration for iridescence is 1,5%.

Nutrients
Nutrients are needed to help the colony grow.

Sources: (Kientz, Vukusic, et al., 2012)(Chapelais-Baron et al., 2018)(Kientz et al., 2012). (Groutars et al., 2022) (Kientz, Ducret, et al., 2012)(Kientz et al., 2013)

2.2.4 Living Aesthetics

Multiple factors exercise influence on the way in which Flavobacteria organise their cells and thus, the colony's structural colour. The way humans experience such changes in Flavobacteria's living colour can be defined as the "living aesthetics" of the colony. This term was proposed by Karana et al. (2020) as the way humans experience the type, degree, and duration of change in a living artefact over time. Understanding the factors that affect the living aesthetics of Flavobacteria is important because it is a significant element in the context of the living label.

Distinct categories are used to describe the living aesthetics of *Cellulophaga lytica*. The research conducted by Groutars and Risseeuw et al. (2022) categorised these variations in Flavobacteria's structural colour, focusing on the characteristic features of form, texture, and iridescent colour. The effect of the environmental stimuli humidity and temperature on a Flavobacteria colony will be described next, guided by the previously mentioned categories. Light will not be mentioned as an environmental stimuli since both growth and iridescence of *C. lytica* are not influenced by light exposure (Kientz et al., 2012).

Humidity

The effect of humidity on colonies of C. lytica has been explored by Groutars and Risseeuw et al. (2022), with the following results:

Form - The size of the colony is influenced by humidity. A higher humidity results in an increase of colony size. The shape of colonies in a humid environment also tend to be more amorphous, while low humidity environments result in a refined circular shape (Figure 2.12).

Texture - High humidity results in a more scattered and pointillistic texture, while low humidity results in a dense and uniform distribution of colour and a smoother texture (Figure 2.12).

Iridescent Colour - The iridescent colour seems slightly more intense at higher humidity (Figure 2.12).

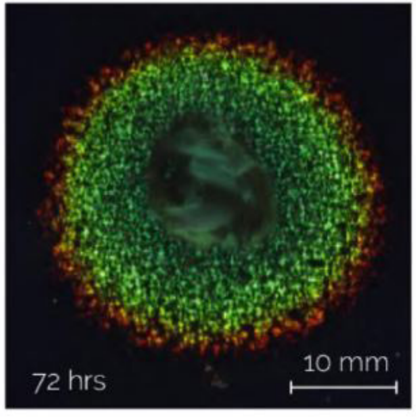
Temperature

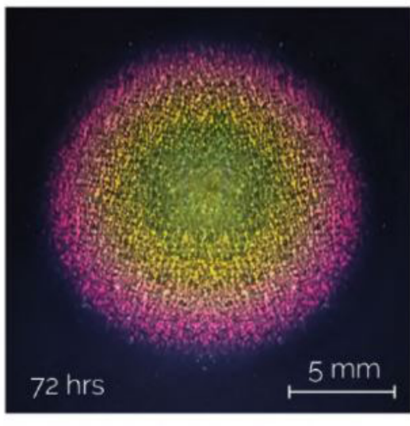
Various researchers have mentioned the effect of temperature on the structural colour of C. lytica (Groutars and Risseeuw et al., 2022;Kientz et al., 2012). A summary of the results is listed below:

Form - *C. lytica* grows slower at low temperatures between 5 and 10 °C (Kientz et al., 2012), therefore the size of the colony is smaller when comparing to higher temperatures (Figure 2.12)

Texture - With a lower temperature, the colony of *C. lytica* seems to be more dense. Initial experiments in this graduation project have also confirmed this (see chapter 4), though it has not been academically documented.

Iridescent Colour - The optimum temperature for iridescence intensity is between 20 and 25 °C or between 5 and 10 °C (Kientz et al., 2012).





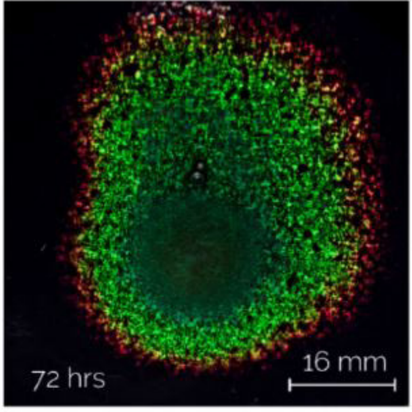


Figure 2.12 - *Standard growth of C. lytica vs. high humidity or lower temperature. Pictures retrieved from (Grouters and Risseeuw et al.)*

To summarise, the effects of environmental stimuli on the living aesthetics of a *C. lytica* colony can be seen in the figure below.

Form

Humidity

Texture

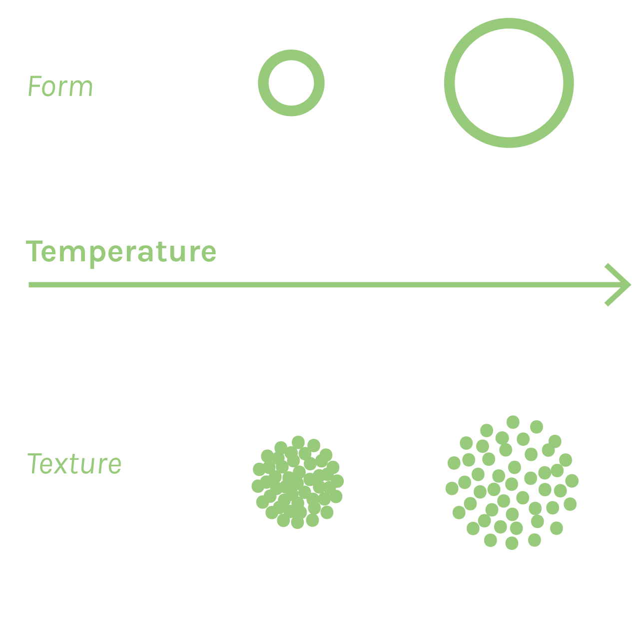


Figure 2.13 *- Effect of temperature and humidity on a Cellulophaga lytica colony*

2.2.5 Design Potential

The concept of using the structural colour characteristic of Flavobacteria to create sensors is not novel. Several researchers have mentioned the potential for Flavobacteria as a sensor like Johansen et al. (2018) and Grouters and Risseeuw et al. (2022):

"Moreover, we envision viable pathways for engineering bacteria toward living sensors with intrinsic self-healing capabilities. As an example, they can be optimised for changing coloration under external stimuli and interface with other living tissues." (Johansen et al., 2018)

"This highlights the potential of these organisms to communicate such changes to us by altering their form, texture and colour, providing a living alternative for digital sensors. Such living sensors will have a relatively slow response but offer new interaction possibilities and expressions compared to digital ones. We present such a possibility with the concept of a living label." (Groutars and Risseeuw et al., 2022)

A picture of the vision for this label can be seen in figure 2.14. These ideas however, have not yet materialised further than a suggestion. Therefore, in this graduation project, the first step will be made towards realizing a design with embedded Flavobacteria. This will then further expand our knowledge on the integration of Flavobacteria into a working concept and create new inspiration for future applications.

Figure 2.14 *- living label by Grouters and Risseeuw et al. Picture retrieved from (Grouters and Risseeuw et al., 2022)*

2.2.6 Flavobacteria in a Flexible Material

The objective of this graduation project is to integrate living Flavobacteria into a flexible and environmentally sustainable material to produce labels. A flexible label enables a familiar interaction for the user (reminiscent of regular packaging labels) while simultaneously creating a habitat for the Flavobacteria outside of the laboratory environment.

The concept of a flexible habitat for Flavobacteria stems from previous experiments involving Flavobacteria embedded in silicone, as illustrated in Figure 2.15 and 2.16. The findings from these experiments confirm that encapsulating Flavobacteria within a flexible material still allows them to develop the necessary structures to generate structural colour.

Nonetheless, the central focus of this project revolves around the creation of a label that is biodegradable. Consequently, silicone is deemed an unsuitable material, necessitating the identification of a biodegradable alternative.

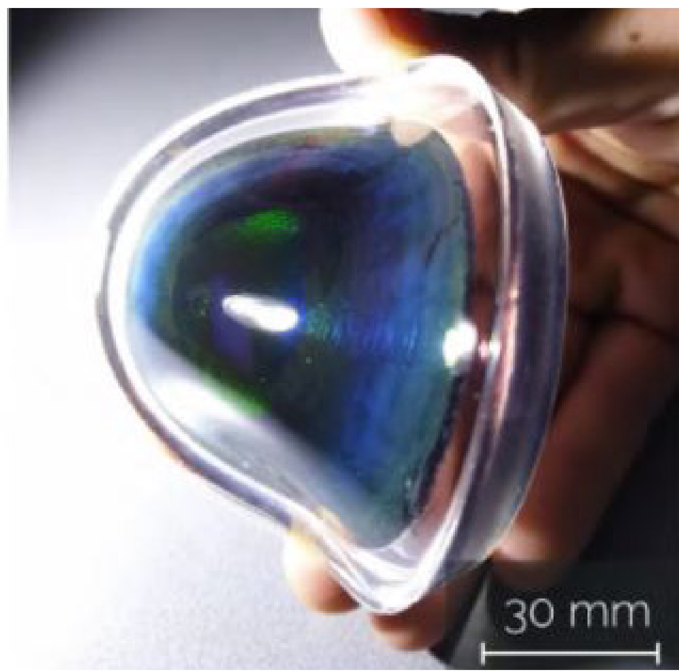


Figure 2.15 *- Flexible habitat with embedded Flavobacteria from silicone. Picture retrieved from (Grouters and Risseeuw et al., 2022)*

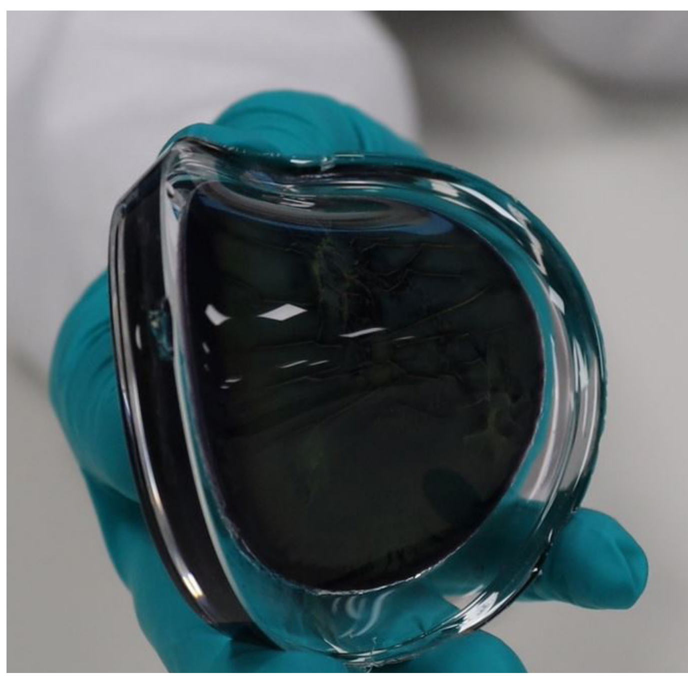
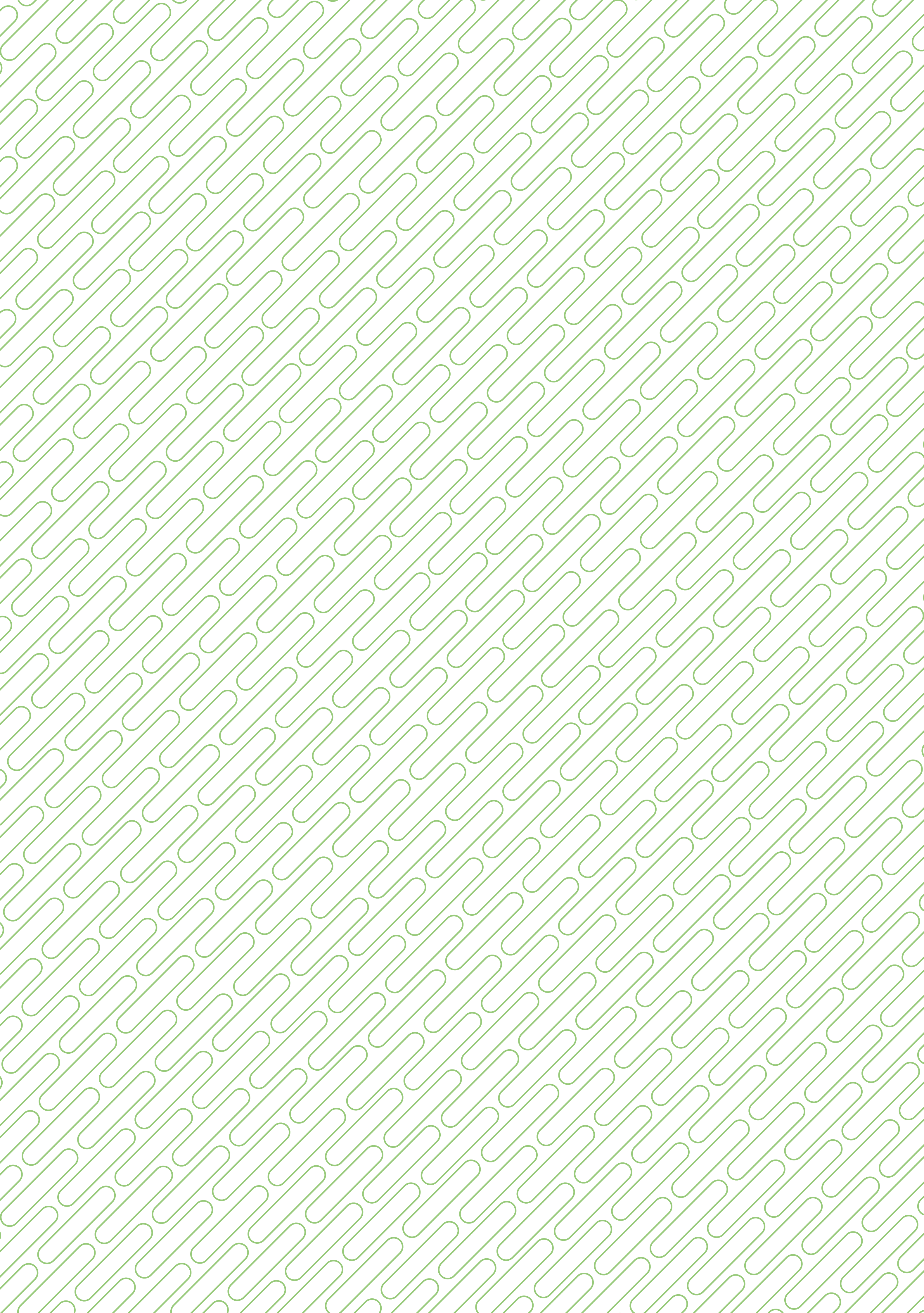


Figure 2.16 *- Personal exploration with Flavobacteria in silicone in previous Biodesign course.*



CH. 3

Getting Started in the BioLab

Introduction

The experiments conducted throughout this project were carried out in the TU Delft IDE BioLab, which is classified as a Biosafety Level 1 laboratory (BSL 1). This classification indicates that the microorganisms studied in this laboratory are typically not known to cause diseases in humans (Coico & Lunn, 2005). The BioLab is equipped with various tools and equipment necessary for conducting experiments with Flavobacteria.

The specific equipment used in the project together with an explanation of how to keep a Flavobacteria stock will be explained in this chapter.

Content

3.1 Equipment **p. 32-33**
3.1.1 Laminar Airflow Cabinet
3.1.2 Freeze Dryer
3.1.3 Capture Tol

3.2 Cultivating Flavobacteria **p. 34-36**
3.2.1 Medium Ingredients
3.2.2 Growth Conditions

Method

Literature research & iterative experimentation

3.1 Equipment

During the experimentation process with Flavobacteria, several equipment were used, including the laminar airflow cabinet, freeze dryer, and capture tool. These instruments are essential for maintaining a sterile environment, preserving samples, and capturing data, respectively. Below, a more detailed explanation of each equipment is provided. Information regarding additional equipment can be found in Appendix 2, which includes a comprehensive list of all the equipment used in the project.

3.1.1 Laminar Airflow Cabinet

Maintaining a sterile environment is crucial for conducting experiments with microorganisms like Flavobacteria. Controlling environmental influences, and minimising the presence of other organisms is essential to ensure accurate and reliable results. The use of a Laminar Airflow Cabinet provides an aseptic working environment that helps prevent contamination. When working with Petri dishes containing Flavobacteria, opening them inside the Laminar Airflow Cabinet minimises the risk of contamination.

3.1.2 Freeze Dryer

The freeze dryer, also known as a lyophilizer, is a piece of equipment used in various experiments throughout the graduation project. It serves the purpose of dehydrating materials for different applications, including long-term storage. For detailed instructions on the specific protocol used for the freeze dryer in this project, Appendix 7.3 can be referred to.

Figure 3.1 - *Laminar airflow cabinet*

Figure 3.2 - *Freeze dryer*

3.1.3 Capture Tool

In this project, all experimental samples were photographed using a Canon EOS D200 camera. The samples were consistently captured from an approximate angle of 45 degrees, with the camera flash serving as the light source. Moreover, a black background was used to provide contrast for the captured images.

Furthermore, in certain instances, the capture tool developed by Clarice Risseeuw was used (Grouters and Risseeuw et al., 2022). This tool facilitates automatic interval capturing of samples, enabling more frequent monitoring of Flavobacteria growth compared to the manual tracking, which can only be done during working hours.

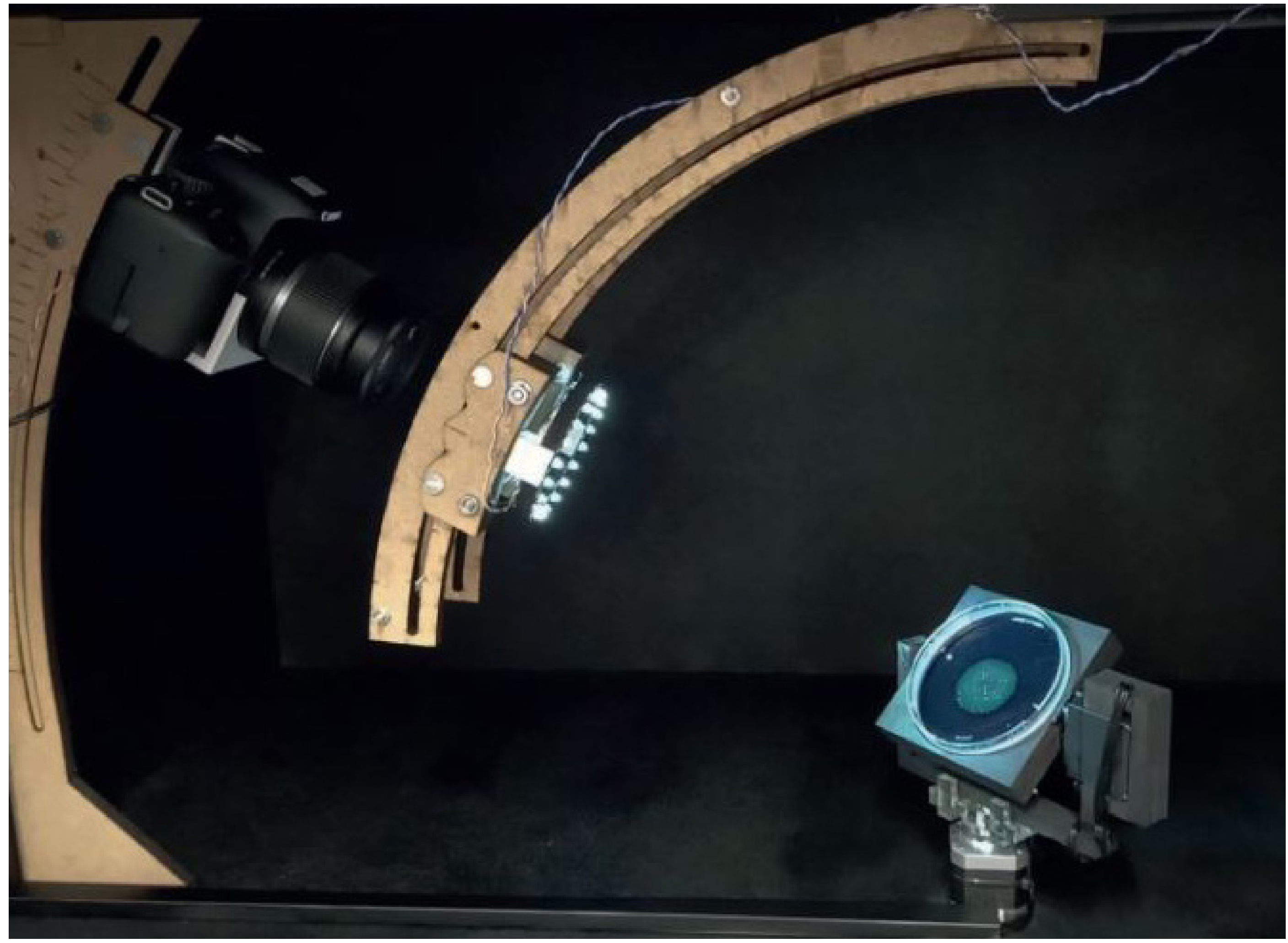


Figure 3.3 - *Capture Tool by Clarice Risseeuw. Picture retrieved from (Risseeuw, 2021)*

3.2 Cultivating Flavobacteria

Given that this graduation project primarily revolves around experimentation with Flavobacteria, maintaining a stock of these microorganisms is essential. The BioLab uses MAR medium as the growth medium for Flavobacteria. MAR stands for Marine, indicating that the medium is specifically designed to support the growth and viability of marine organisms such as Flavobacteria. This medium can be used in both liquid and solid states (Figure 3.4) and is prepared in the BioLab (Figure 3.5).

To ensure a constant supply of young and active Flavobacteria cells for experiments, a weekly transfer process is implemented. This involves transferring the Flavobacteria onto fresh Petri dishes containing MAR medium.

3.2.1 Medium Ingredients

As previously discussed in chapter 2.2.3, in order to grow Flavobacteria need oxygen, seawater components and nutrients. The MAR medium therefore consists of the following elements:

Yeast and Peptone (Nutrients)
Sea Salt, MgSO4, KNO3 (Salinity)
Demi Water (Humidity)

The water in the medium not only provides humidity but also serves as a diluent for the components of the medium. The combination of these ingredients is also referred to as MAR "liquid medium" and is mostly used to culture Flavobacteria. In order to maintain a long-term stock of Flavobacteria, a preservation method is used whereby the Flavobacteria are suspended in liquid medium along with glycerol and subsequently frozen at -80°C. This freezing process ensures the viability and longevity of the Flavobacteria for future use.

Figure 3.4 *- Solid (left) and liquid (right) MAR medium*

Figure 3.5 *- Making MAR medium in the BioLab*

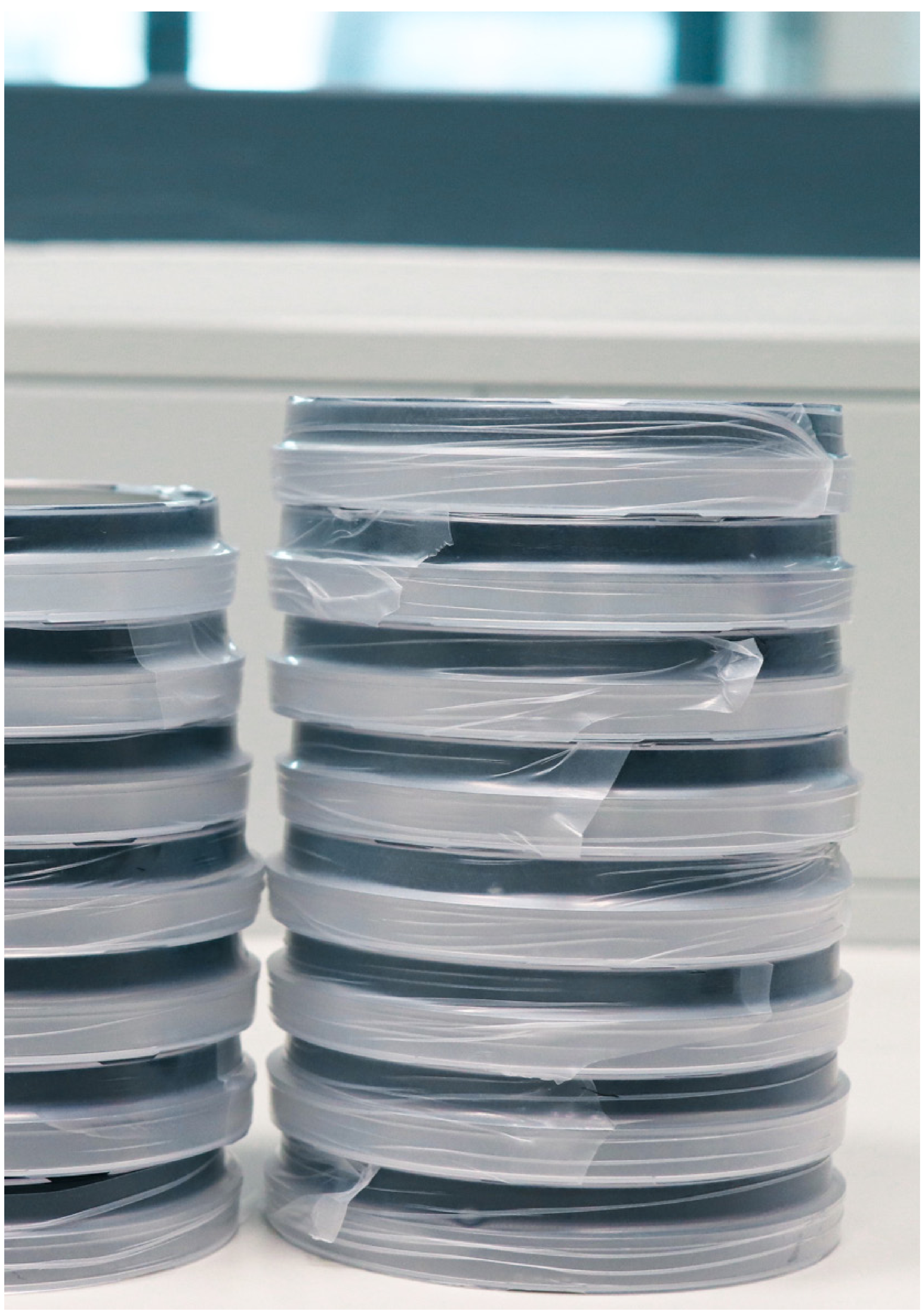


Figure 3.6 *- Petri dishes with MAR medium sealed with Parafilm*

Since Flavobacteria need a semi-solid surface to make structural colour, agar was added to the medium as a gelling agent. Additionally, to establish a dark background that enhances the contrast with the structural colour of the Flavobacteria, the black pigment nigrosine is utilised. The combination of the standard MAR medium ingredients with the agar and nigrosine can be referred to as "Solid medium" and is poured into petri dishes after sterilisation to create this semi-solid sterile surface. To prevent the medium from drying out, the Petri dishes are securely sealed with parafilm, as illustrated in Figure 3.6. In the instance that "MAR medium" is discussed in this report, the "solid medium" is referred to unless specifically stated as liquid MAR medium. The ingredients for both the liquid and solid medium can be seen in Figure 3.7. The exact amount of all the ingredients and the method of preparation can be found in Appendix 3.

Figure 3.7 *- Ingredients for MAR medium*

As mentioned earlier, the weekly Flavobacteria stock is also grown on solid MAR medium in Petri dishes. It is interesting to note that within these stock plates, slight variations in the medium composition, growing conditions, or preparation method can lead to differences in the living aesthetics of Flavobacteria. These variations can manifest in variations in colony size, density, and even iridescence, as demonstrated in Figure 3.8.

3.2.2 Growth Conditions

In this project, the majority of experiments were carried out under standard conditions, which entail maintaining room temperature and utilising MAR medium following the standard recipe. Henceforth, whenever "standard conditions" are mentioned, it refers to these specified conditions.

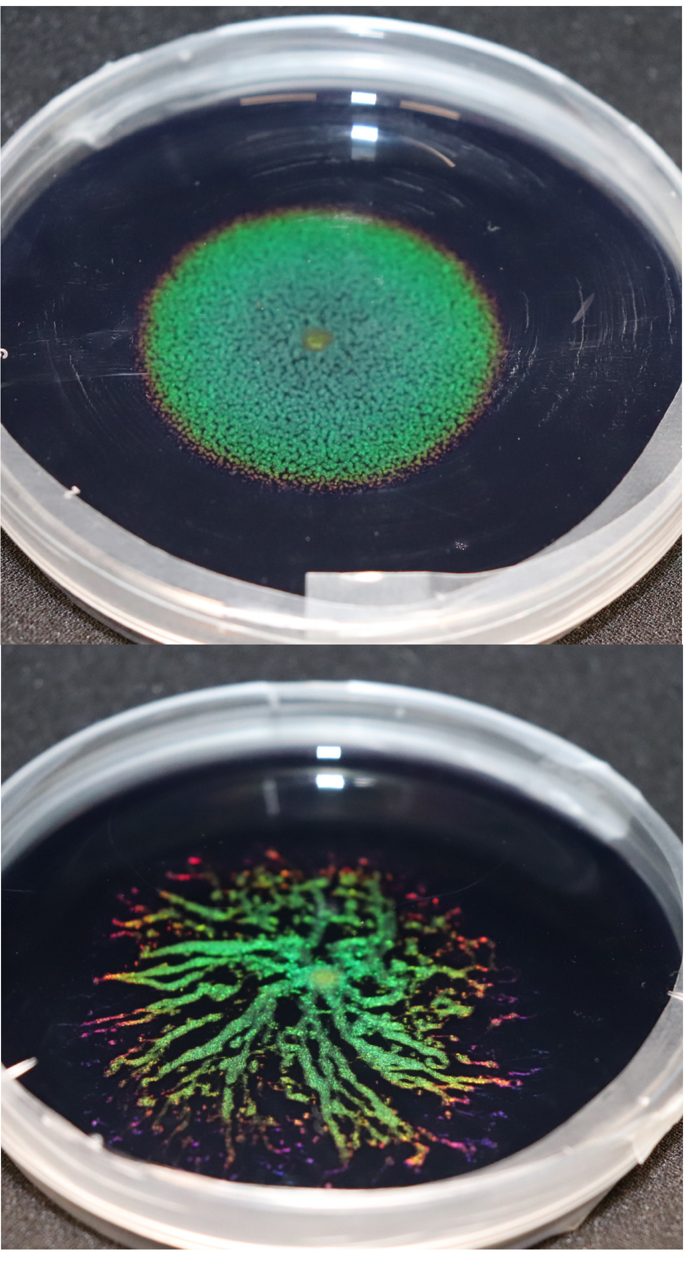
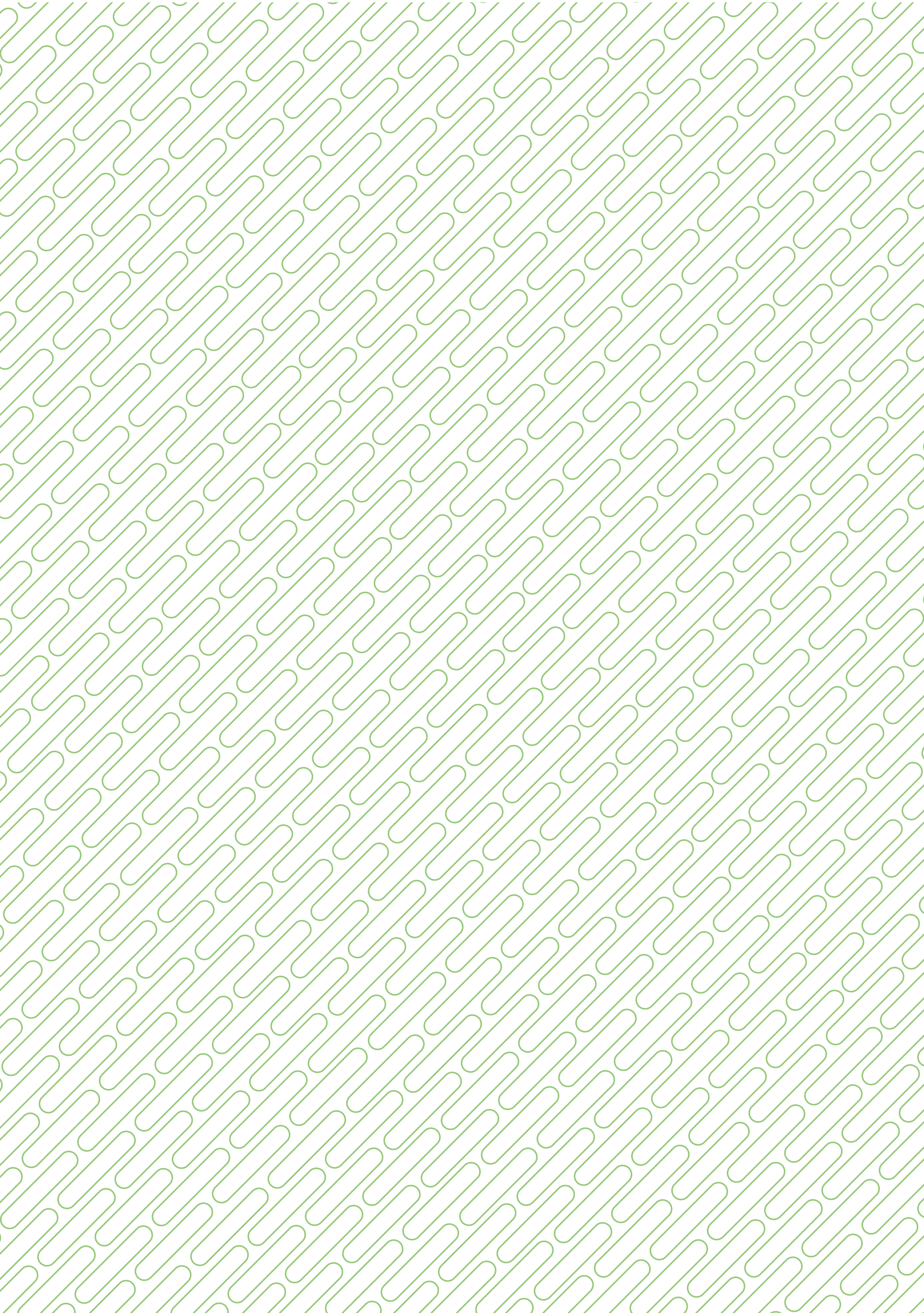
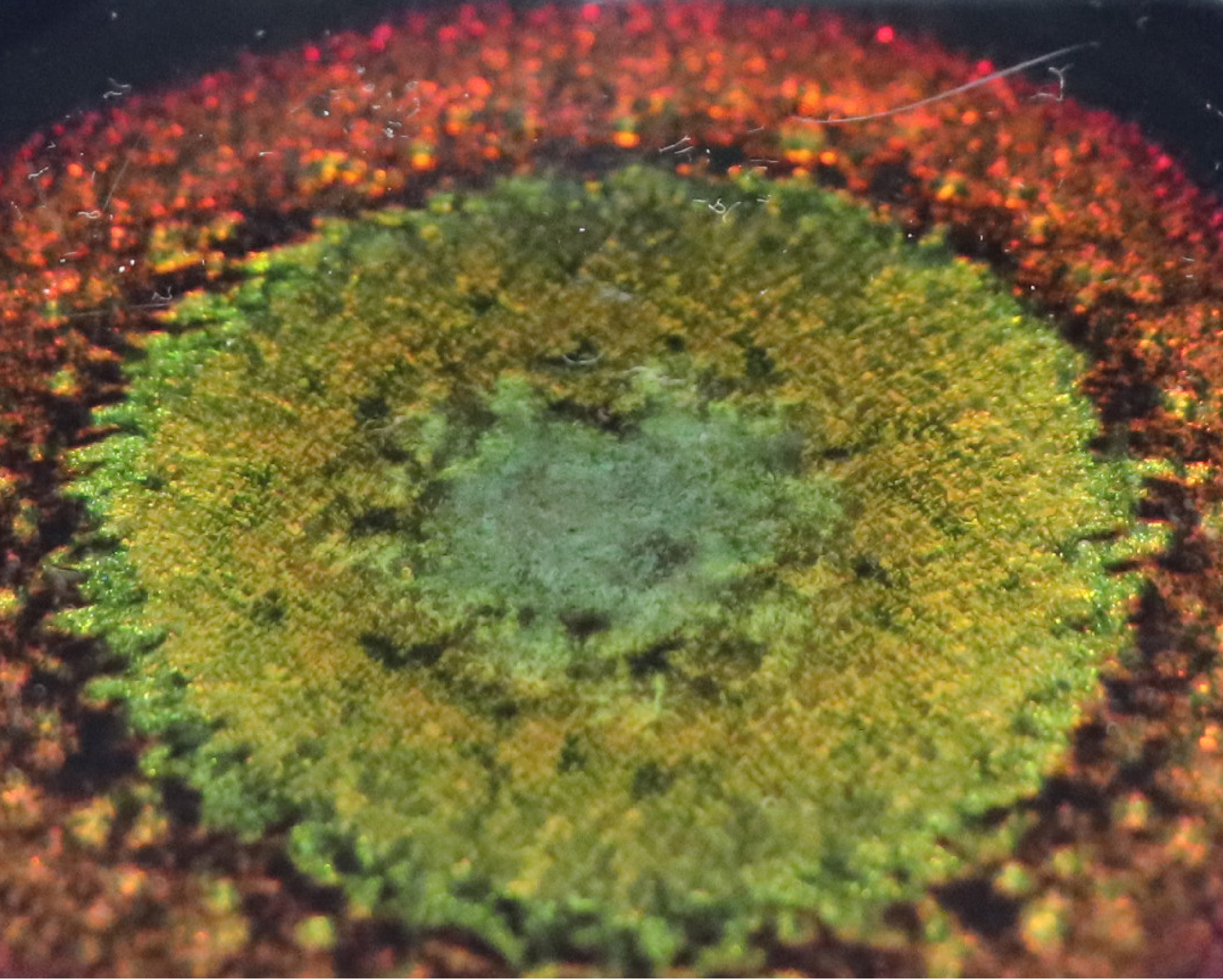


Figure 3.8 *- Cellulophaga lytica stock plates, grown in standard conditions*





CH. 4

Sensor Validation

Introduction

Flavobacteria are able to produce iridescent structural colour, which has considerable potential for communication applications due to its responsiveness to environmental stimuli. Therefore, Flavobacteria are proposed as a sensor in this project.

In the context of this particular project, the ability of Flavobacteria colonies to show different living aesthetics in response to temperature variations will be referred to as their "communicative potential." In this chapter, this communicative potential of Flavobacteria will be validated.

Content

4.1 Temperature Sensor — **p. 40-41**

4.2 Response of *C. lytica* — **p. 42-44**

4.2.1 Temperature Rings
4.2.2 Colony Diameter
4.2.3 Iridescent Colour
4.2.4 Application

Method

Literature research & iterative experimentation

4.1 Temperature Sensor

The way in which different environmental stimuli can change the living aesthetics of Flavobacteria is described in chapter 2.2.4. Flavobacteria show change in iridescent colour at low temperatures (Figure 4.1 and 4.2) and also grow significantly slower than in standard conditions at room tempertature (RT), as can be seen in figure 4.3. Therefore, taking the form, texture and iridescent colour into account, temperature was chosen as the environmental stimuli to design for. This choice was additionally motivated by the ease of temperature transfer, as the label can remain sealed without requiring any additional modifications in the design to let the environmental stimuli through.

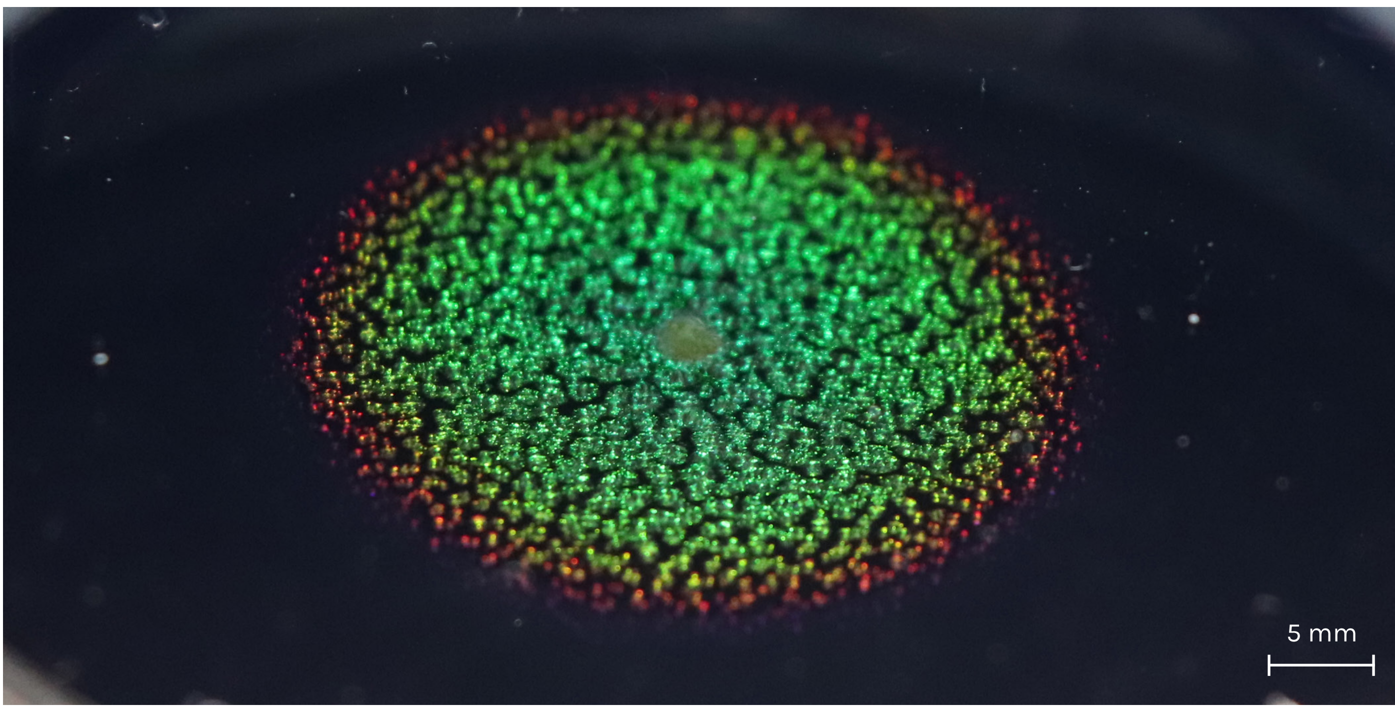


Figure 4.1 *- Cellulophaga lytica grown on MAR medium for 3 days, standard conditions*

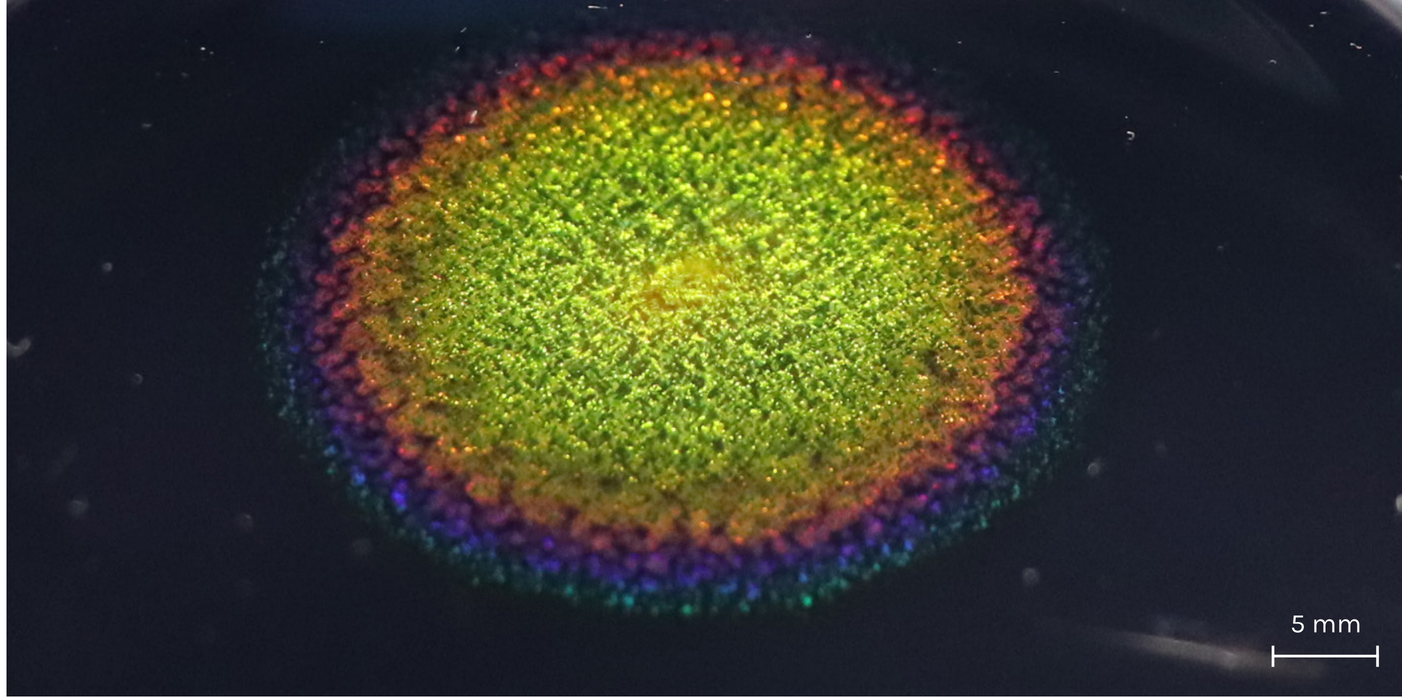


Figure 4.2 *- Cellulophaga lytica grown on MAR medium for 31 days, in fridge (4 °C)*

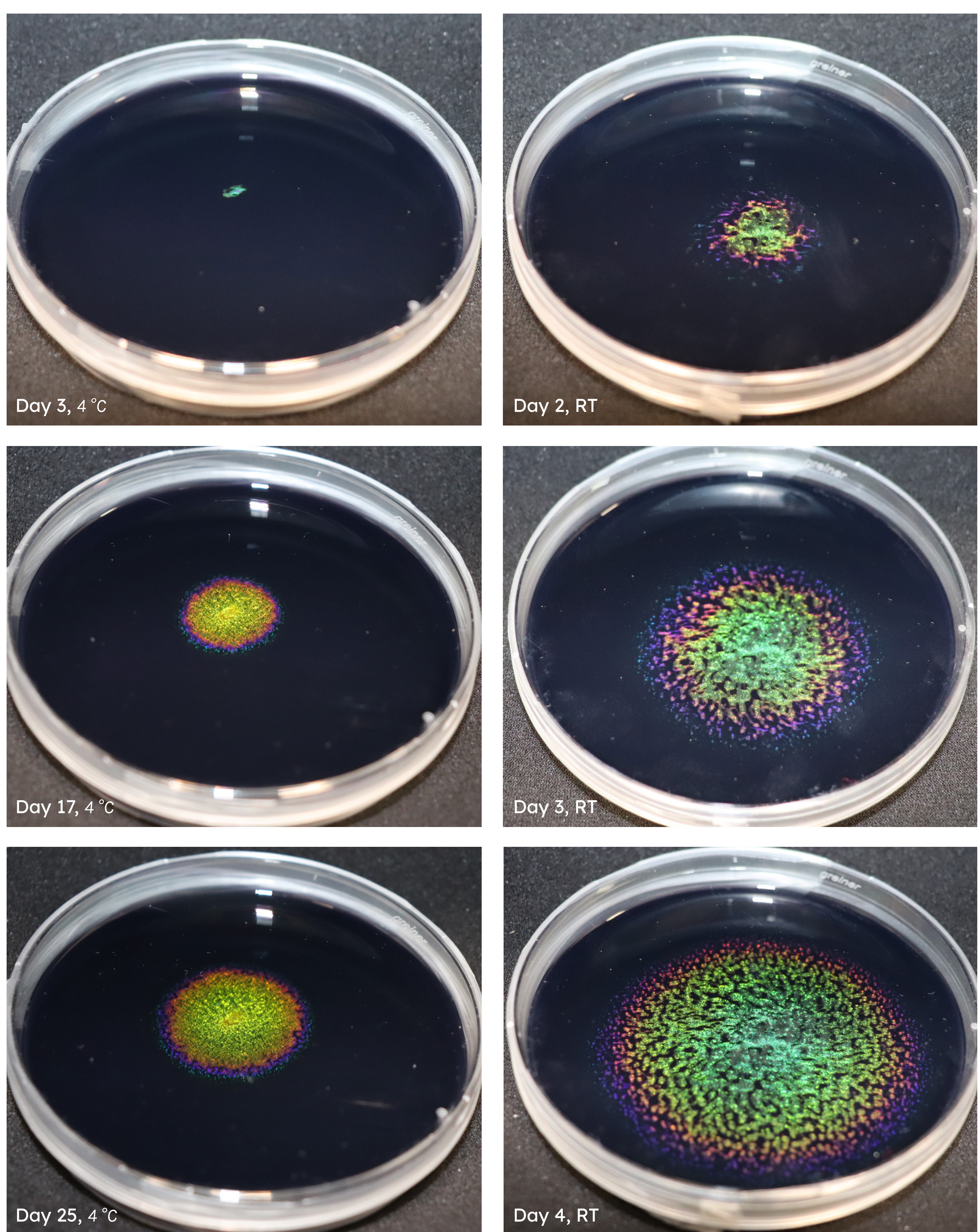


Figure 4.3 *- Cellulophaga lytica grown on MAR medium, growth comparison between fridge (4 °C) and standard conditions*

4.2 Response of *C. lytica*

Before starting the design process to create a label that accommodates for the environmental stimuli of temperature, a validation of the communicative value of *C. lytica* was conducted. It is known how *C. lytica* grows in cold temperatures compared to optimal temperatures, however it was unclear what happens when this microorganism is grown in alternated temperature regimes.

Therefore, *C. lytica* was grown in MAR medium Petri dishes (x3) for 24 days, with different temperature intervals: room temperature and 4°C (See Figure 4.4) Supplementary data can be found in Appendix 4.

4.2.1 Temperature Rings

From the conducted experiment, it could be seen that circles in the colony appear with a temperature change. They do not disappear when growth continues, creating a sort of temperature log. These rings are reminiscent of the growth rings of a tree (Figure 4.5).

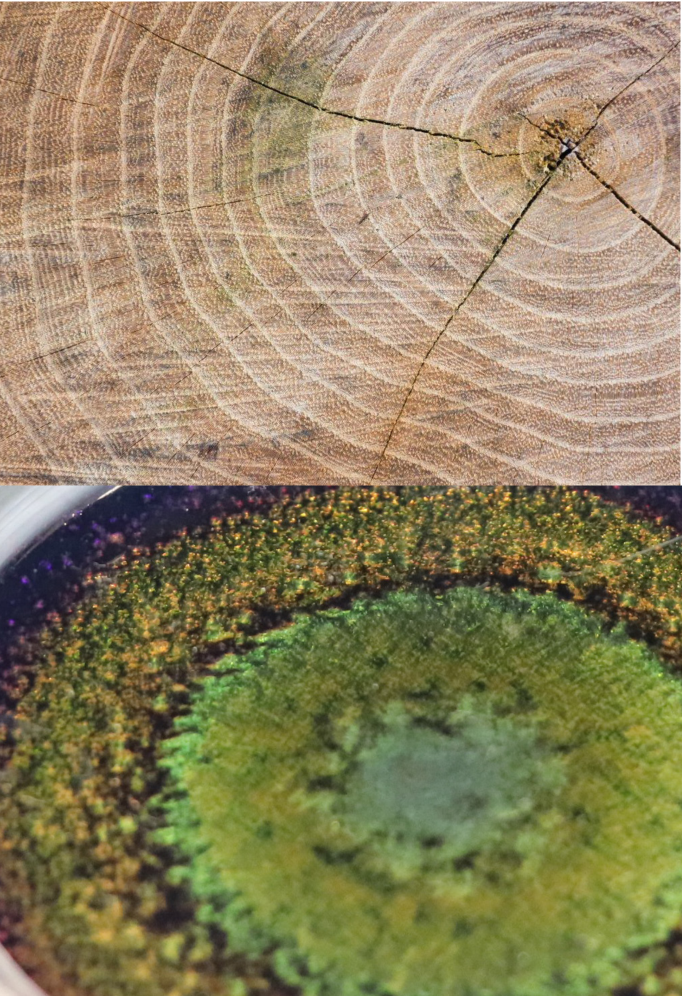


Figure 4.5 - Growth rings of a tree (Jaarringen | Ecopedia, n.d.) compared to temperature rings in *C. lytica*

Day 1-3
Room Temp. (3 days)
(Pictures
taken on day 3)

Day 3-14
Fridge (11 days)
(Pictures
taken on day 14)

Day 14-15
Room Temp. (1 day)
(Pictures
taken on day 15)

Day 15-23
Fridge (8 days)
(Pictures
taken on day 23)

Day 23-24
Room Temp. (1 day)
(Pictures taken on
day 24)

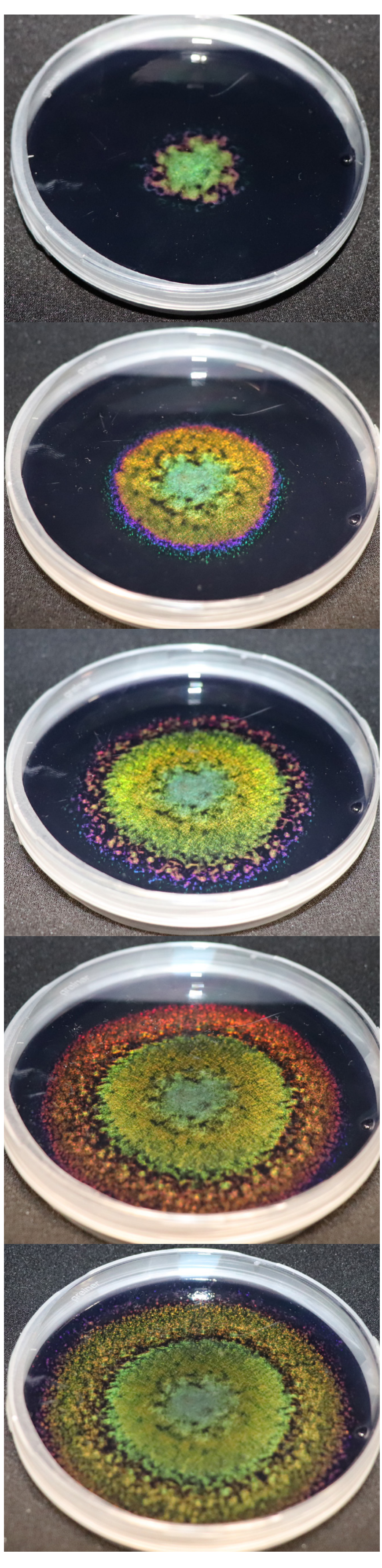


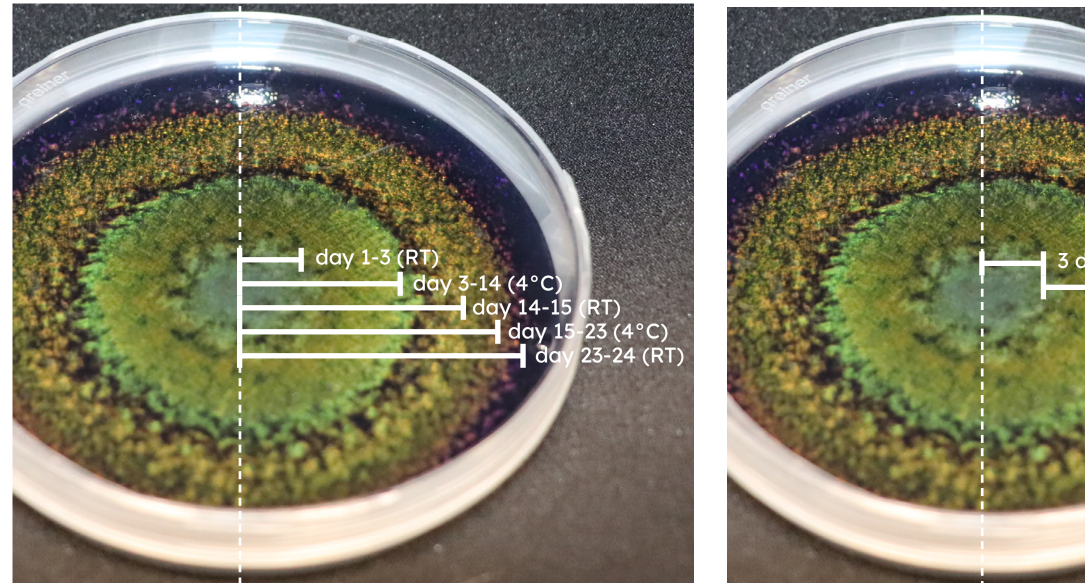
Figure 4.4 - *C. lytica* on MAR medium in alternated temperature regimes

4.2.2 Colony Diameter

As predicted the expansion of the colony under colder temperatures was a lot slower and therefore, visibly, it is possible to determine if the Flavobacteria have been submitted to lower (fridge) temperatures, or not, based on colony size (Figure 4.6).

4.2.3 Iridescent Colour

Another observation that can be made are the variations in iridescent colour. Figure 4.7 illustrates that the section of the colony grown in the fridge displays an orange/red hue, whereas the portion cultivated at room temperature appears more green. As previously explained, the colony's outer edges exhibit lower cell density and contain younger cells, leading to mostly red hues. In the case of cells grown in the fridge, this same phenomenon can be observed due to the lower cell density in that region. Conversely, the parts that grow at room temperature show more of a green colour. This could be attributed to the colony's faster growth rate at room temperature, resulting in higher cell density in the initially refrigerated area and subsequently altering the iridescent colour (Figure 4.7). However, despite these colour changes, the temperature rings remain intact, thereby allowing for continued visibility of the temperature logging information.



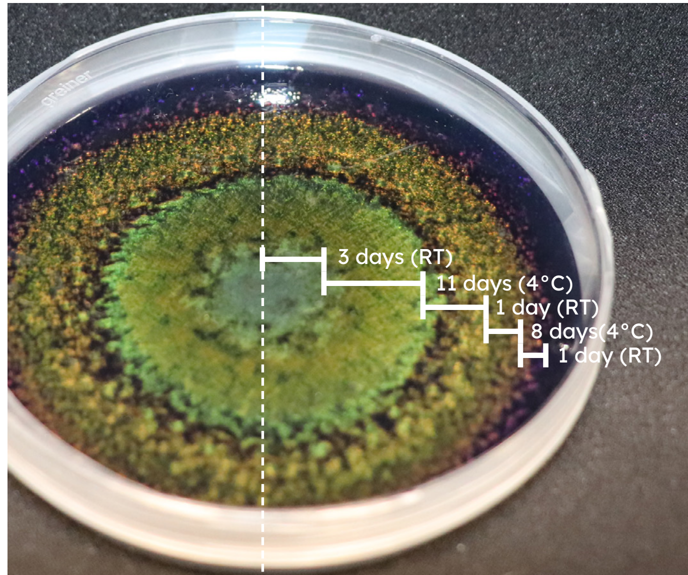
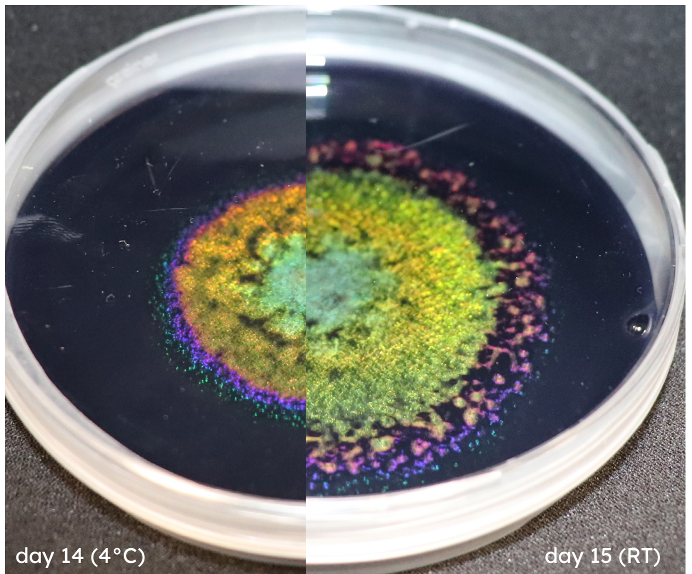


Figure 4.6- Colony diameter expansion under standard conditions (room temperature) and fridge temperature (4 °C)



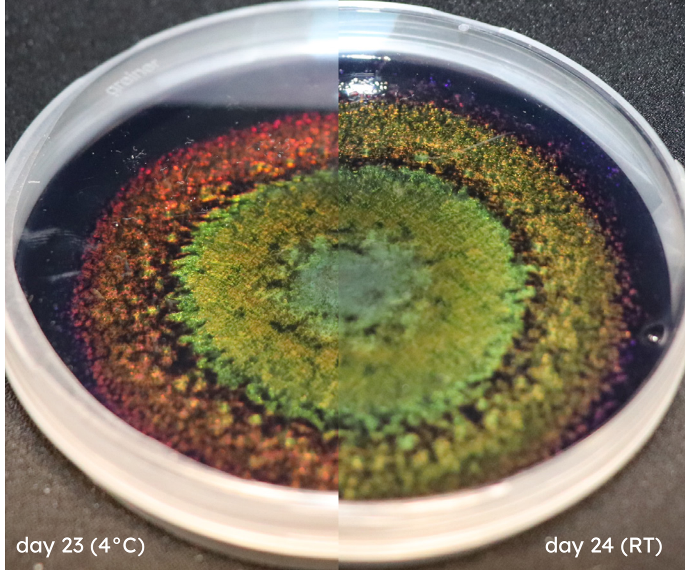
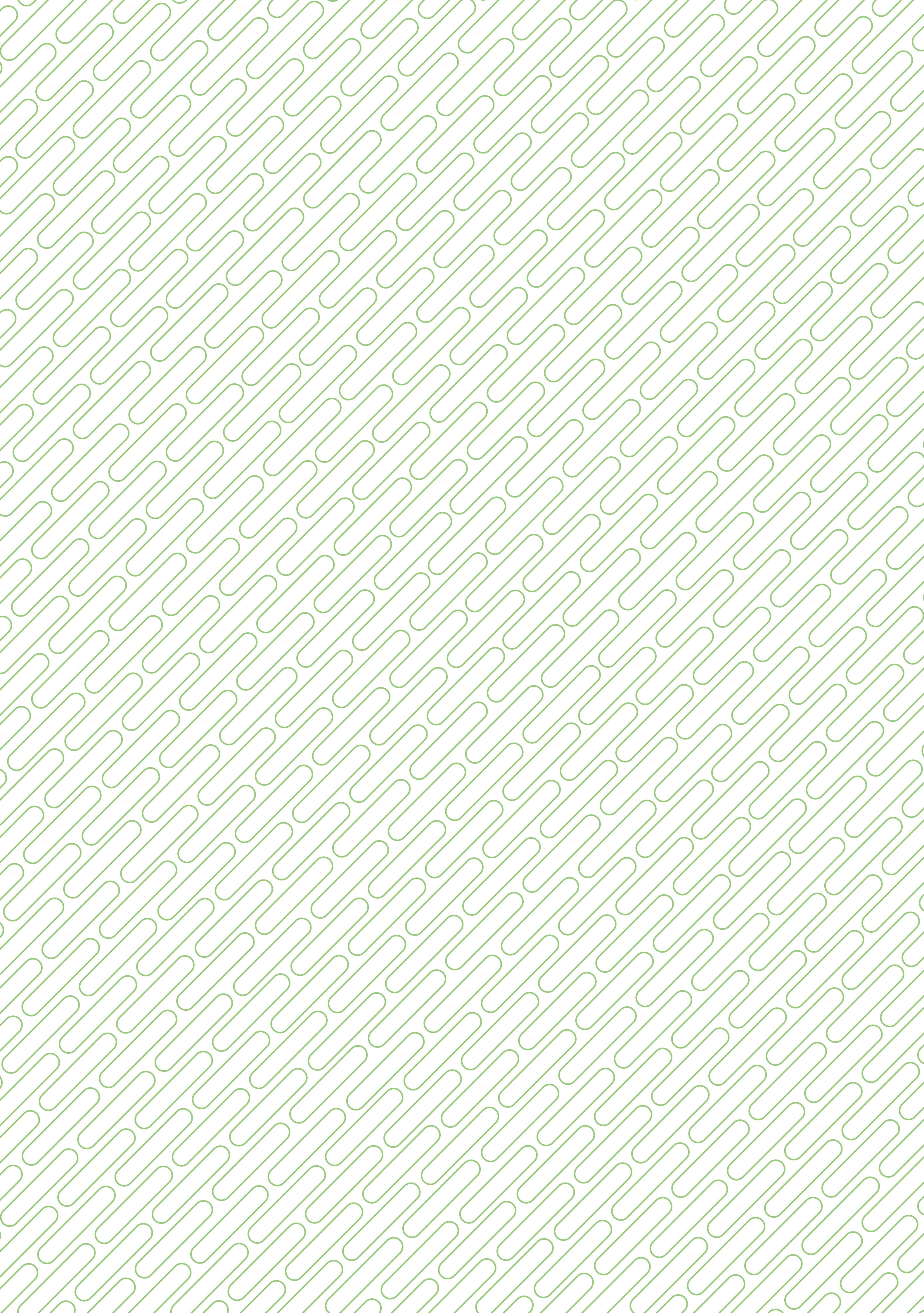
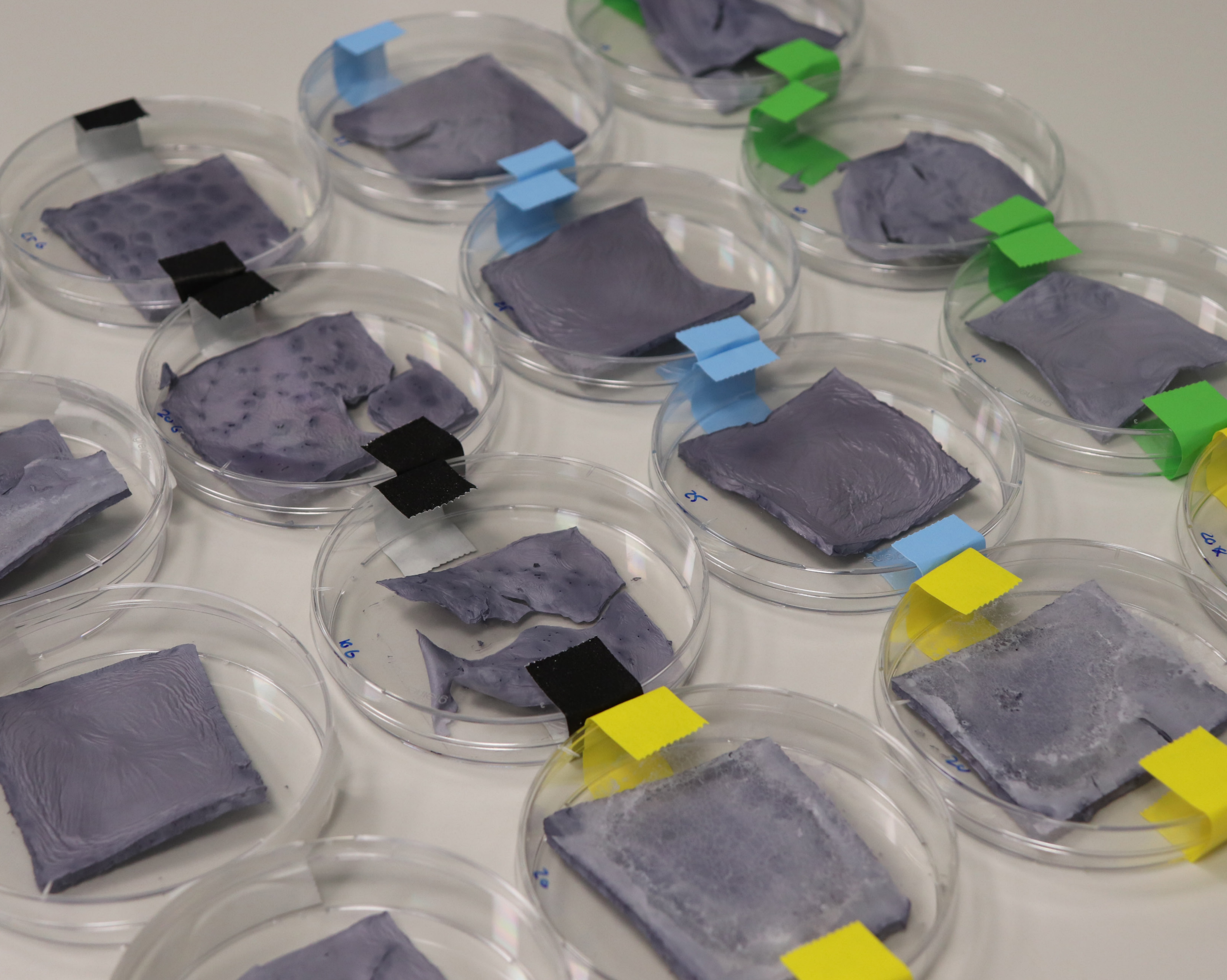


Figure 4.7- Iridescence of the temperature rings of *C. lytica* after temperature fluctuations

4.2.4 Application

Considering the communicative value of Flavobacteria, not only do temperature-induced changes affect the living aesthetics of the bacterial colony, a “record” is also kept of the temperature history with the temperature rings and the colony diameter. This characteristic makes Flavobacteria highly suitable for applications involving products that require consistent temperature maintenance over extended periods of time. Furthermore, the size of the label can be customised to accommodate specific use cases. Since it is known that under standard conditions Flavobacteria expand outward at an approximate rate of 5 mm per day when cultivated in a petri dish (Groutars and Risseeuw et al., 2022), it becomes possible to determine the label’s size based on the desired time limit for a product to remain outside refrigeration.





CH. 5

Design Process

Introduction

The design requirements established for the living label form the basis of the iterative design process in this project. These requirements were formulated through a combination of literature research and the validation of Flavobacteria's sensing capabilities, as discussed in the previous chapter. They are outlined in this chapter and then translated into challenges. These are then linked to the various experiments conducted in this graduation project.

Content

5.1 Design Requirements **p. 48**

5.2 Experiments **p. 49**

Method

Iterative experimentation

5.1 Design Requirements

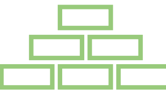
The requirements can be divided into two distinct categories: those applicable to Flavobacteria and those applicable to the materials used in the design.

Cellulophaga lytica

C. lytica should be able to make structural colour in the living label.

C. lytica should be able to interact with temperature changes within the label and communicate them via their living aesthetics.

C. lytica should be contained within the label so that they will not grow on surfaces outside of the product.



Materials

The entire label should be biodegradable and preferably incorporate one or more biodegradable materials from Bio4Life.

The material of the top layer should be transparent, enabling the visibility of the structural colour beneath it.

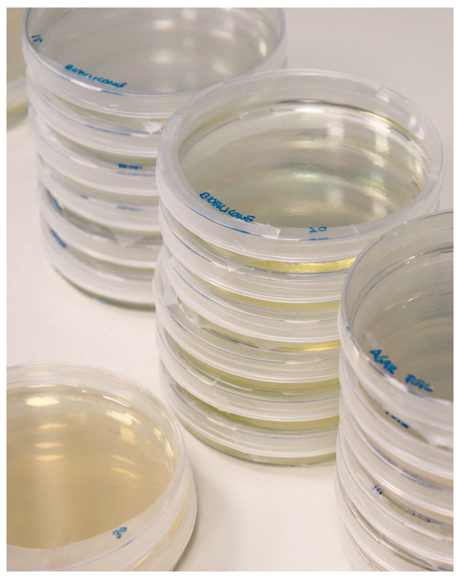
The living label should be flexible enough to bend.

The living label should allow the transference of temperature changes to the embedded Flavobacteria in the living label.

5.2 Experiments

The three iteration directions defined in chapter 1.3 (the top layer, the activation method and the alternative medium) can be translated into three different challenges to tackle in this graduation project. These can be seen in Figure 5.1. Each challenge resulted in a series of experiments that led to the living label proposed at the end of this report. An overview of the different experiments done in each direction that are mentioned in this report can be found in Figure 5.1. The following chapters will further explain the experimentation done in each iteration direction.

Top Layer
Finding a material that C. lytica can make structural colour under



Experiments

Bio4Life materials
(PLA and Natureflex)

Hydrophobic coating
(beeswax)

Biomaterials
(agar foil and bio silicone)

Physical headspace

Activation Method
A way to activate the growth of C. lytica

Experiments

Freeze drying Flavobacteria

Alternative Medium
An alternative to the standard MAR medium that is more suitable for the living label

Experiments

MAR medium
(without nigrosine)

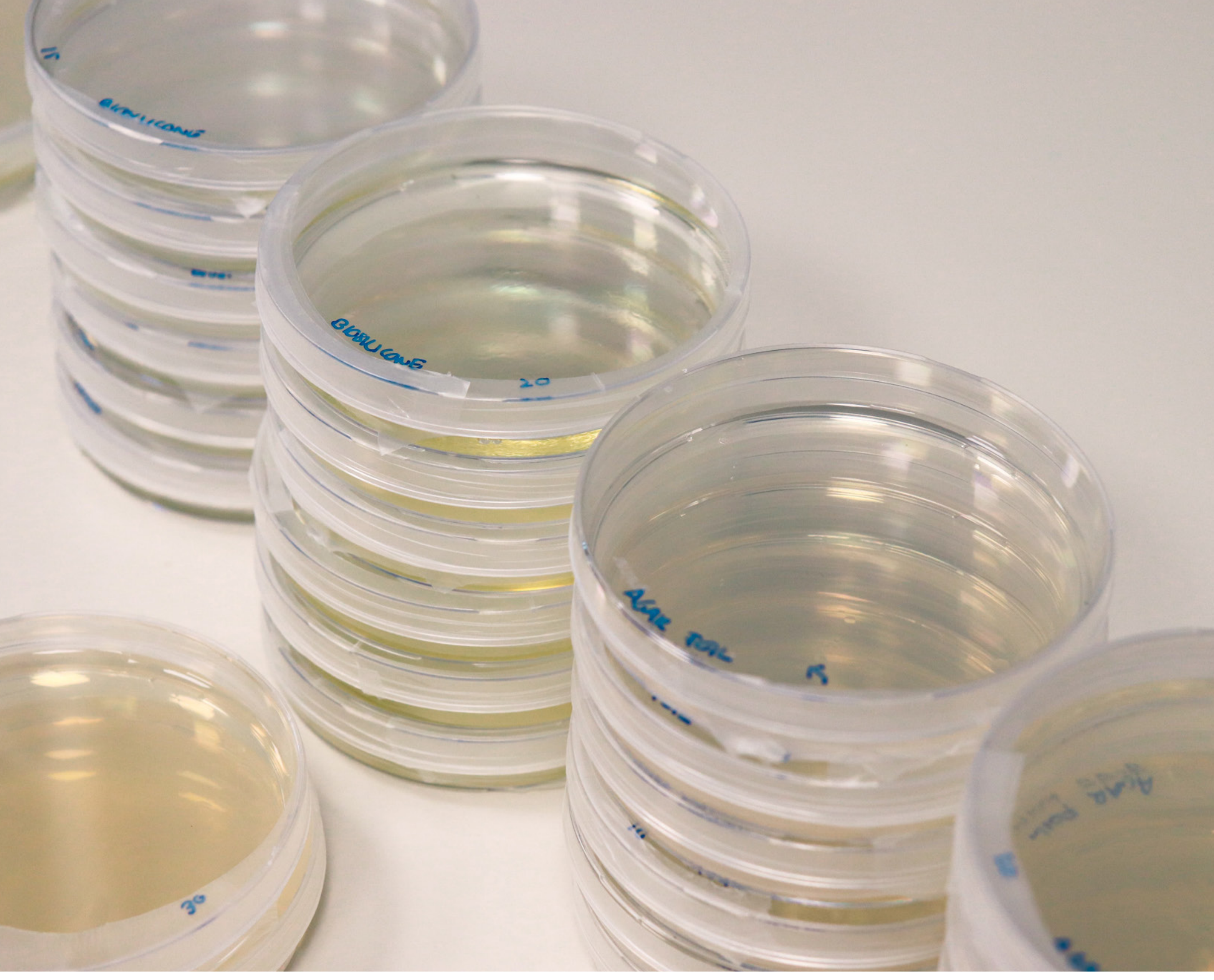
Calcium Alginate
(Freeze-dried and normal)

Bio4Life Materials
(PaperWise)

Polyvinyl alcohol (PVA)

Freeze drying MAR medium

Figure 5.1 - *Iteration directions with corresponding challenges and experiments*



CH. 6

Top Layer

Introduction

The most crucial part of the living label is the iridescence resulting from the structural colour formed by Flavobacteria. It is therefore essential that the top layer of the label enables the generation of this structural colour. In this chapter, biodegradable materials will be explored for the top layer of the label.

Content

6.1 Starting Point p. 52

6.2 Bio4Life Materials p. 53

6.3 Hydrophobic Coating: Beeswax p. 54

6.4 Biomaterials p. 55
6.4.1 Bio Silicone
6.4.2 Agar Foil

6.5 Headspace p. 60-63

Method

Iterative experimentation

6.1 Starting Point

Experiments from a previous course on design with living artefacts and initial experiments of this graduation project have shown that *C. lytica* is able to make structural colour under silicone (Figure 6.1 and 6.2). Both silicone that was still liquid (not hardened yet) as can be seen in Figure 6.1, and silicone after curing (Figure 6.2) did not seem to disturb the cell organisation of *C. lytica*. To our knowledge, silicone is the only material known to enable the development of this unique structural colour by *C. lytica*. In this project however, the aim is to create a biodegradable label. Therefore, in this chapter biodegradable materials will be explored to find a suitable option for the top layer.

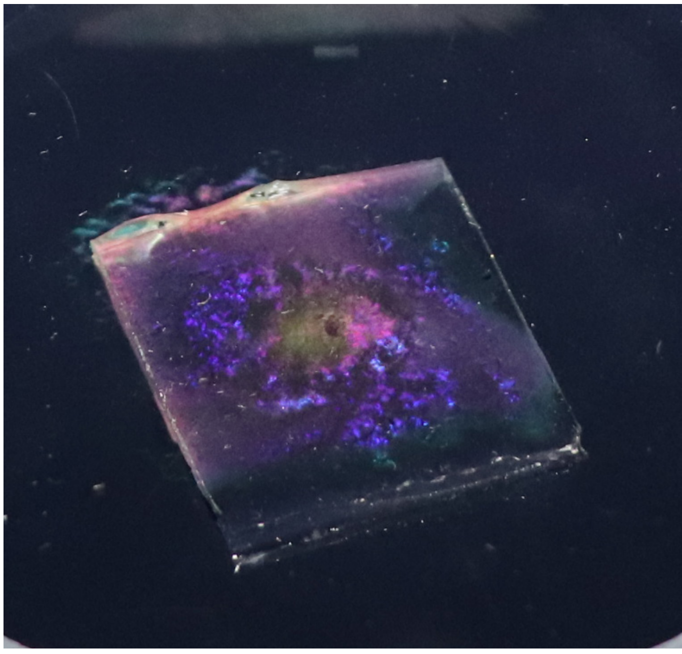


Figure 6.2 - *Cellulophaga lytica under silicone (solid, Placed on top)*

Figure 6.1 - *Cellulophaga lytica under silicone (Liquid before hardening, Poured over)*

6.2 Bio4Life Materials

Bio4Life has several biodegradable materials that were tested in this project. During the assessment of materials from Bio4Life for the top layer of the living label, two transparent options were considered: PLA and NatureFlex. PLA is derived from renewable resources such as corn starch, tapioca, or sugarcane, while NatureFlex is made from cellulose (Bio4life - Supplier of Biodegradable Labels, 2022). Notably, these materials are fully certified for (industrial) compostability according to the EN13432 standard.

To determine the suitability of PLA and NatureFlex as the top layer of the living label, Flavobacteria, taken from the stock plate after several days of growth, were inoculated with a sterile loop on MAR medium, and both materials were placed on top straight after inoculation. The strain was then cultivated at standard conditions for a period of 5 days (Figures 6.3 and 6.4). The results indicate that neither PLA nor NatureFlex from Bio4Life facilitated the production of structural coloration when placed directly on the Flavobacteria colony. However, the presence of the materials did not hinder the growth of Flavobacteria, as evidenced by their appearance at the edges of the material. Therefore, based on these findings, it can be concluded that PLA and NatureFlex from Bio4Life are unsuitable as the top layer of the living label when directly placed on the medium.

In addition to PLA and NatureFlex, various other materials from Bio4Life were tested in the same way. This was done to understand the behaviour of Flavobacteria in combination with these materials and to determine possible applications for them within the living label. However, since these materials were not transparent, they were excluded from this chapter as they would not be suitable for the desired application of the top layer. For a comprehensive overview of these experiments, as well as the complete documentation of the PLA and NatureFlex experiments, please refer to Appendix 5, 6.1 and 6.2.

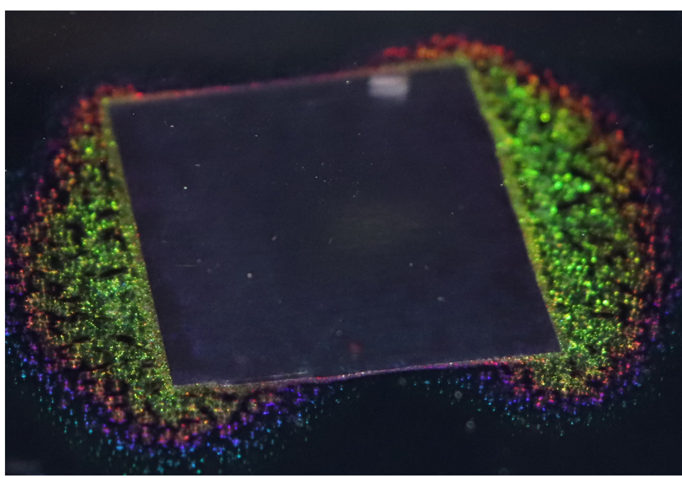


Figure 6.3 *- C. lytica colony under NatureFlex after 5 days of growth, standard conditions*

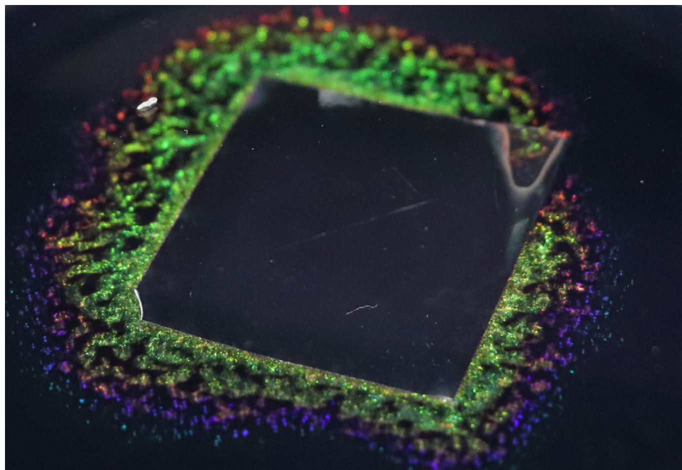


Figure 6.4 *- C. lytica colony under PLA after 5 days of growth, standard conditions*

6.3 Hydrophobic Coating: Beeswax

Based on the findings of the previous experiment, it can be concluded that *C. lytica* does not produce structural colour when grown under any of the Bio4Life materials tested. To better understand the following steps in the search for a suitable top layer for the living label, a deeper understanding of the properties that make silicone suitable for the formation of structural colour was necessary. When analysing silicone as a material, it was noted that one characteristic enabling the formation of structural colour could be the hydrophobic nature of silicone (Figure 6.5). In light of this, further research was conducted to explore biodegradable alternatives to hydrophobic materials. This investigation aimed to identify materials that possess hydrophobic properties similar to silicone while also being environmentally friendly and biodegradable.

In order to test the hypothesis regarding the role of hydrophobicity in the formation of structural colour, PLA material from Bio4Life was coated with beeswax and positioned directly over Flavobacteria on MAR medium. These Flavobacteria were taken from the stock plate after several days of growth, and inoculated with a sterile loop on the MAR medium. Beeswax was chosen for this experiment since it is known for its natural hydrophobic properties (Figure 6.6). Figure 6.7 displays the outcome after 4 days of growth in standard conditions.

From this experiment it can be seen that it was not the beeswax that enabled structural colour, but rather the space that had emerged from the shape of the material (Figure 6.7). This seems to indicate that hydrophobicity of a material is not the (only) requirement needed for Flavobacteria to make their structural colour under that certain material. The full documentation of this experiment can be found in Appendix 6.4 and 6.5.

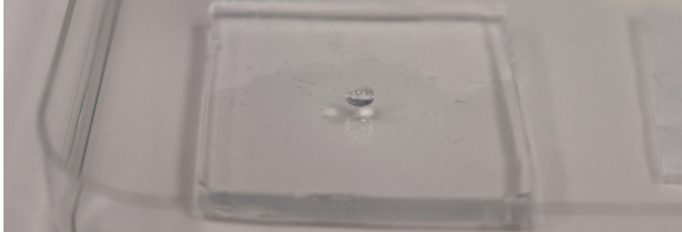


Figure 6.5 *- Water droplet on silicone*

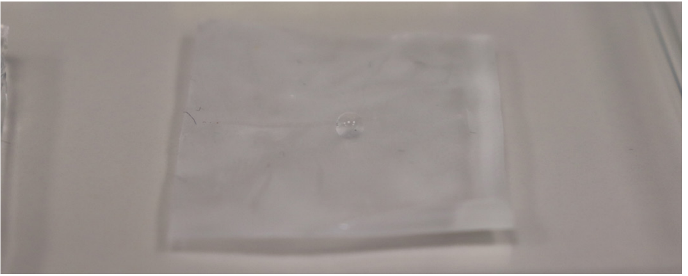


Figure 6.6 *- Water droplet on PLA covered in beeswax*

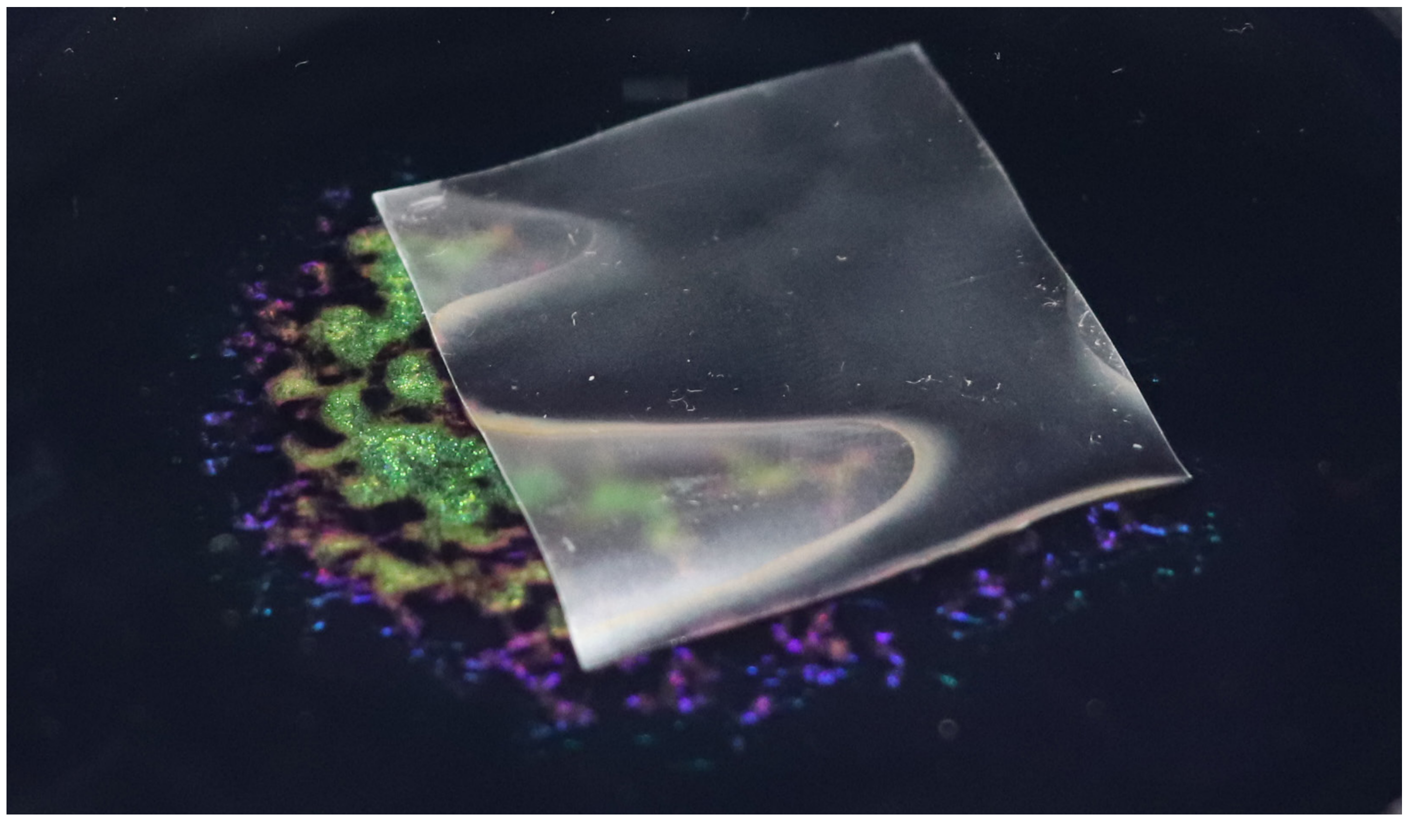


Figure 6.7*- C. lytica growing under PLA covered in Beeswax, day 4, standard conditions*

6.4 Biomaterials

Since hydrophobicity alone may not be the sole characteristic responsible for enabling Flavobacteria to form structural colour, further investigation was conducted to explore other potential properties of silicone that contribute to this behaviour. Consequently, this subchapter focuses on the experimentation with biodegradable alternatives to silicone.

After researching various biomaterials with properties resembling those of silicone, bio silicone (Figure 6.8) and agar foil (Figure 6.9) were selected for further evaluation. The primary factors considered in their selection were transparency, which is crucial for the top layer of the label, and a behaviour that closely resembled that of regular silicone.

The preparation process for these biomaterials follows a similar principle. It involves the combination of a polymer, a plasticizer, and water to dissolve and blend the polymer and plasticizer (Bogers, n.d.). The recipes utilised for both the bio silicone and agar foil were based on the methodology developed by Loes Bogers (Bogers, n.d.). The complete preparation methods can be found in Appendix 6.6 and 6.7.

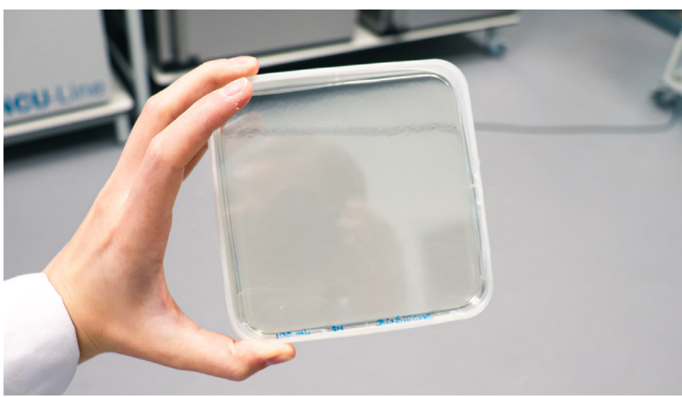


Figure 6.8 *- Bio silicone in Petri Dish*

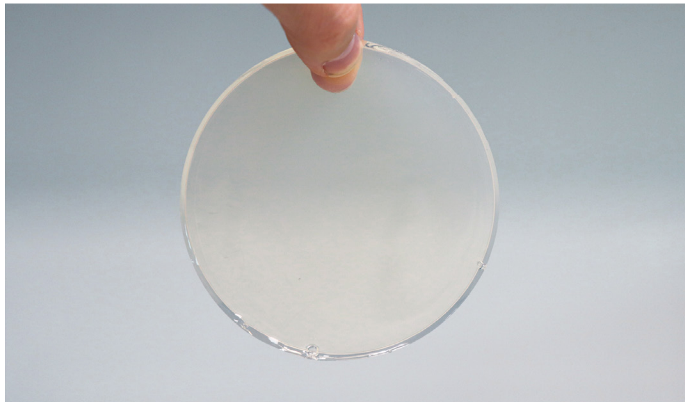


Figure 6.9 *- Agar foil*

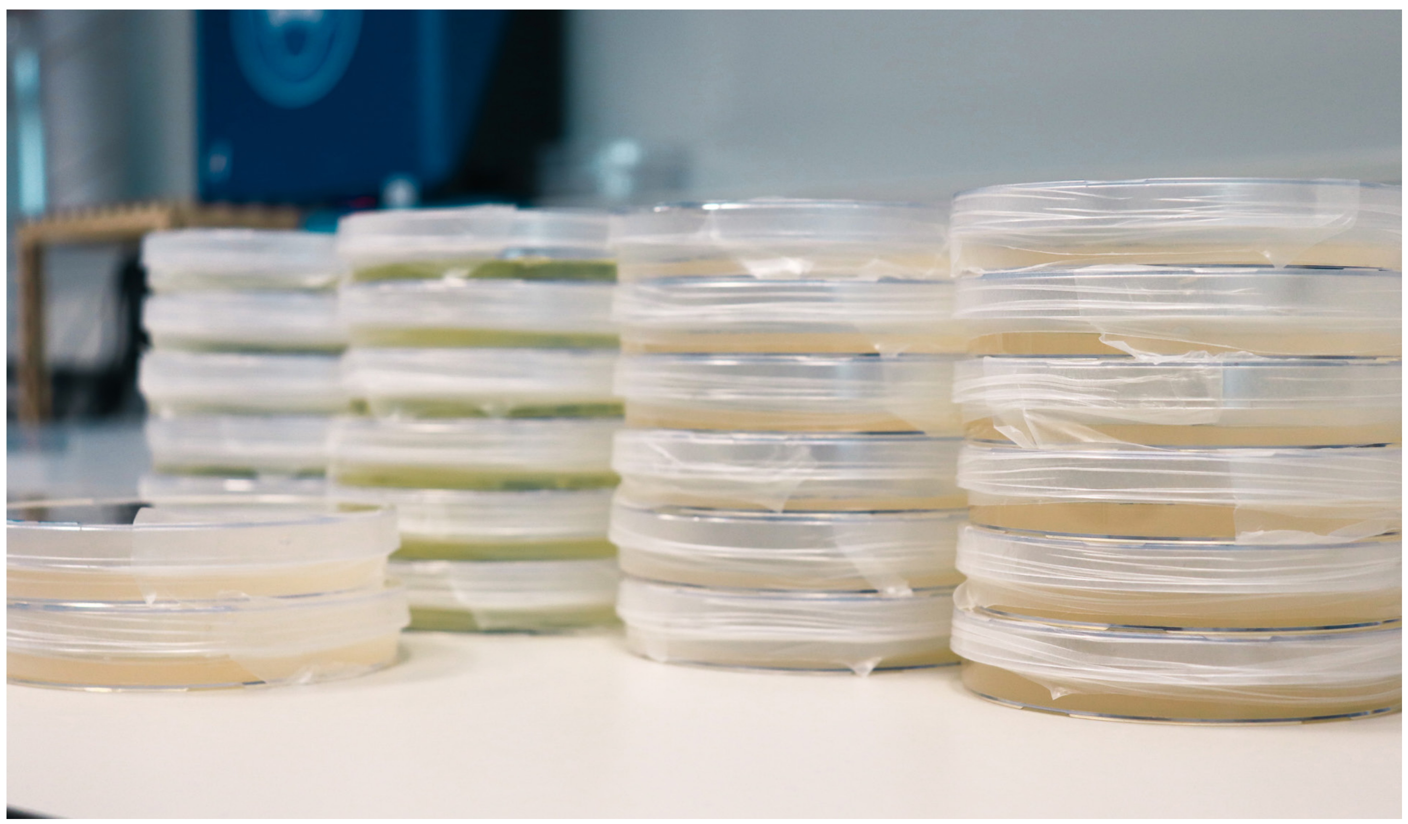


Figure 6.10- Bio silicone and agar foil prepared in Petri dishes

6.4.1 Bio Silicone

Bio silicone consists of three ingredients: gelatine, glycerin and water. In this case the gelatine functions as a polymer to make sure the material becomes a solid and the glycerine is the plasticizer to make sure it stays flexible. Bio silicone seems to be hydrophobic after preparation (Figure 6.11).

Despite its visual resemblance to silicone, bio silicone did not resemble silicone in its behaviour when attempting to remove it from the dish. It was found to be brittle and prone to breaking, resulting in the collection of only small fragments (Figure 6.12). When placed over a Flavobacteria colony immediately after inoculation, no structural colour was observed under the bio silicone after 4 days of growth in standard conditions. However, the presence of structural colour at the edges of the material indicated that Flavobacteria growth was possible beneath the bio silicone (Figure 6.13 and 6.14). Interestingly, the structural colour adjacent to the bio silicone displayed a pink/purple iridescence. This indicates that bio silicone does seem to affect the structural colour of Flavobacteria. Additionally, after 9 days of growth, the bio silicone exhibited signs of degradation (Figure 6.15). Considering these observations, it can be concluded that bio silicone is not a suitable material for the top layer of the living label.

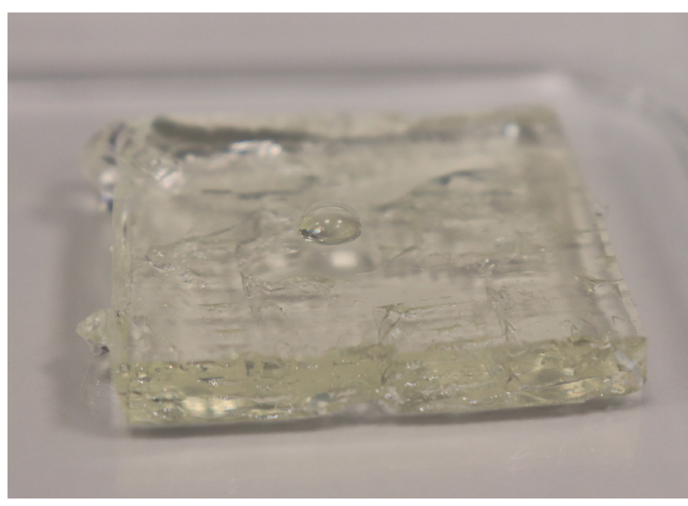


Figure 6.11 - *Water droplet on bio silicone*

Figure 6.12 - *Bio silicone parts*

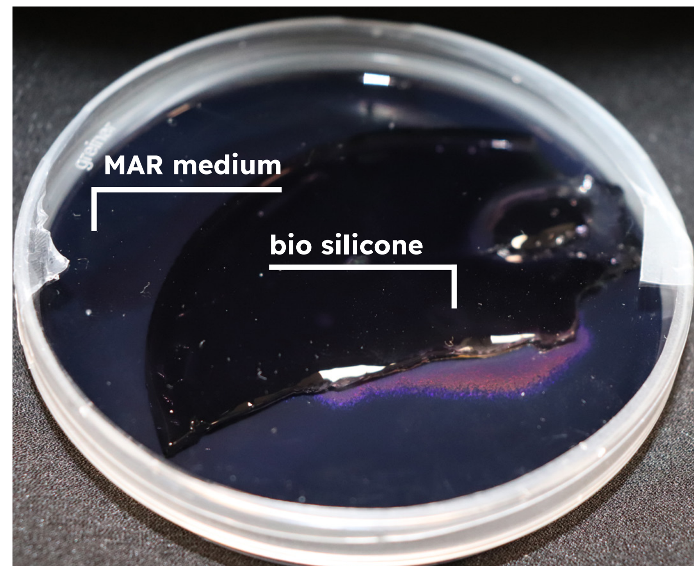


Figure 6.13 - *C. lytica colony under bio silicone, 4 days, standard conditions*

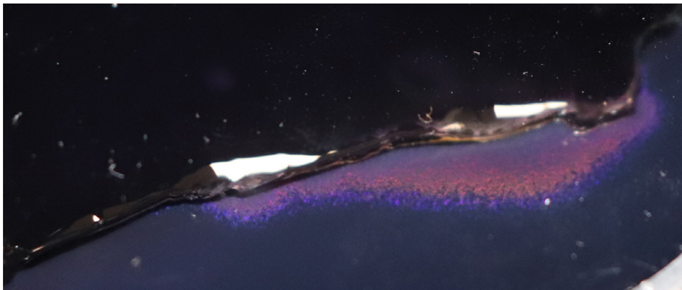


Figure 6.14 - *C. lytica colony under bio silicone, 4 days, standard conditions (close-up)*

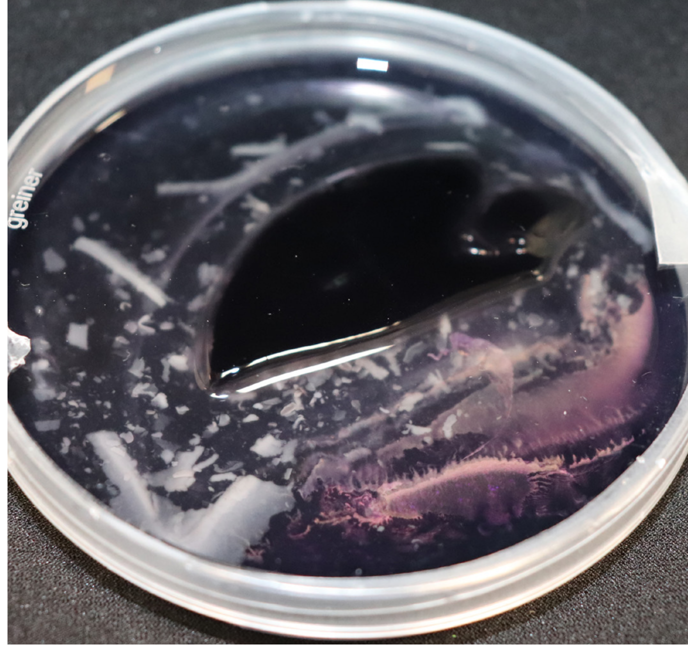


Figure 6.15 - *C. lytica colony under bio silicone, 9 days, standard conditions*

6.4.2 Agar Foil

Another potential biodegradable alternative to silicone is agar foil, which is composed of agar, glycerine, and water. Agar serves as the polymer to solidify the material, while glycerine acts as the plasticizer to maintain flexibility. Unlike silicone, agar foil does not exhibit hydrophobic properties (Figure 6.16).

It was observed that this material closely resembles regular silicone in terms of its appearance. Notably, the shape of the agar foil remains intact even after bending, indicating its durability and resilience as can be seen in Figure 6.17.

When agar foil was placed over a Flavobacteria colony immediately after inoculation, no structural colour was observed under the agar foil after 5 days of growth in standard conditions (Figure 6.18 and 6.19). However, it was observed that Flavobacteria were able to grow beneath the agar foil, as evidenced by the presence of structural colour at the edges of the foil where it was slightly lifted from the MAR medium on which the colony was growing (Figure 6.19). Notably, the structural colour that appeared in the space between the agar foil and the MAR medium exhibited predominantly red and purple hues. This indicates that the agar foil does affect the structural colour formed by the Flavobacteria.

While agar foil may not be suitable as a direct top layer for Flavobacteria, it still possesses valuable qualities such as its silicone-like feel and flexibility. Therefore, it should be considered for other components of the label design.

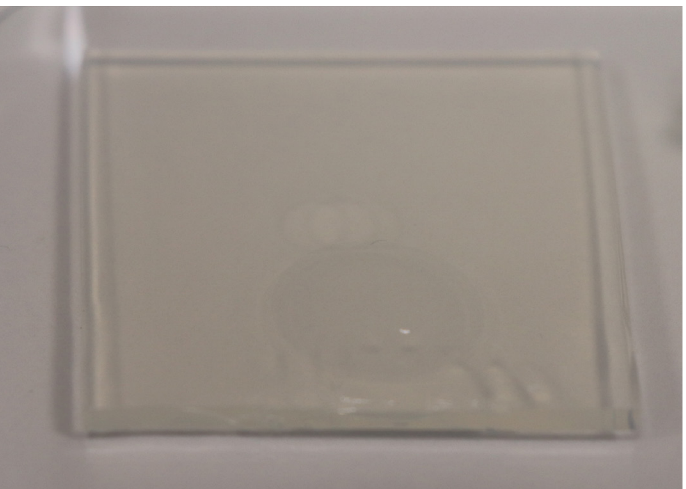


Figure 6.16 - *Water droplet spread out on agar foil*

Figure 6.17 - *agar foil taken out of the Petri dish*

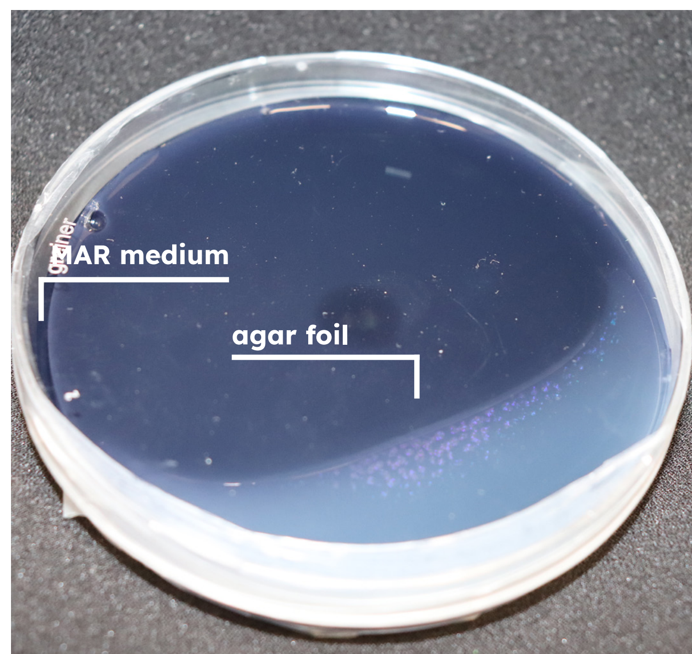


Figure 6.18 - *C. lytica colony under agar foil, 5 days, standard conditions*

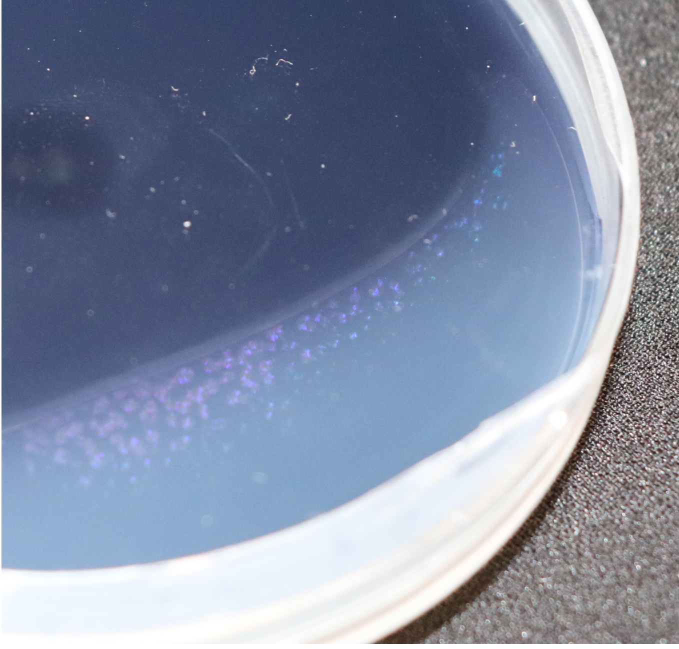


Figure 6.19 - *C. lytica colony under agar foil, 5 days, standard conditions (close-up)*

6.5 Headspace

In the previous experiments with biodegradable materials, it was shown that Flavobacteria only seem to form structural colour when physical space exists between them and the top layer. Therefore, In order to allow Flavobacteria to produce structural colour while still being enclosed, a construction was devised to create physical headspace (Figure 6.20). The materials used for this purpose were agar foil (as described in Chapter 6.4.2) and transparent PLA material (as discussed in Chapter 6.2). These headspaces were placed on a petri dish containing MAR medium inoculated with Flavobacteria from day 1. As depicted in Figure 6.21, it is evident that structural colour was successfully formed under the headspace. Notably, the headspace did not appear to affect the growth of the colony, as its size was comparable to that of the control sample (Figure 6.22).

An attempt was also made to create headspace using a combination of agar foil and NatureFlex material from Bio4Life. However, this construction did not maintain sufficient transparency. Further details and results can be found in Appendix 6.8.

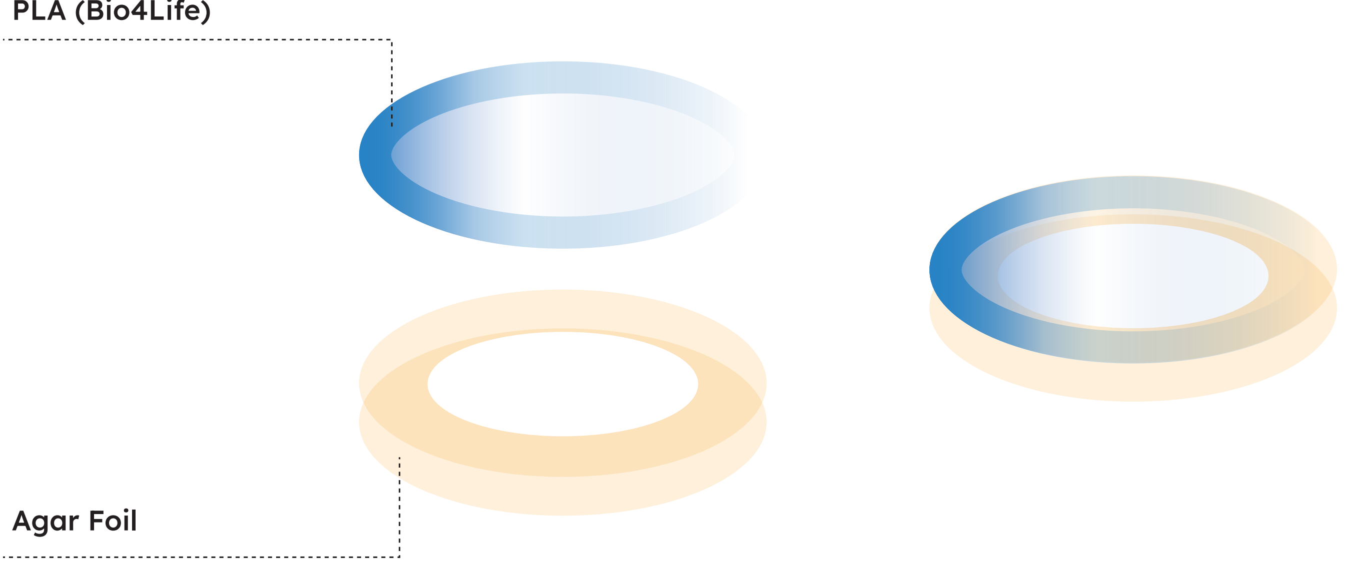


Figure 6.20- *Exploded view of physical headspace construction*

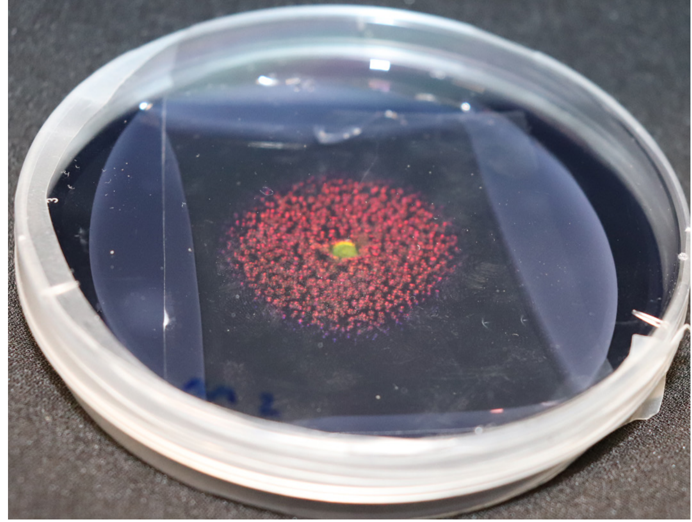


Figure 6.21 - *C. lytica colony grown under physical headspace, day 3, standard conditions*

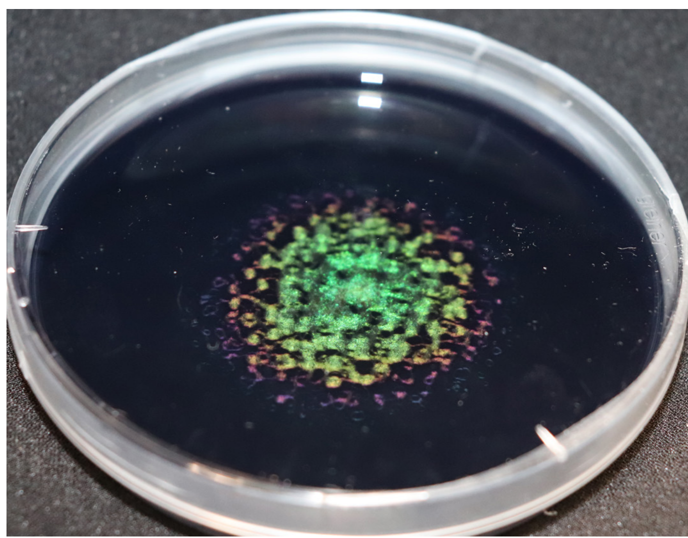


Figure 6.22 - *C. lytica control sample, day 3, standard conditions*

Furthermore, the compostable BioTak adhesive from Bio4Life was tested to evaluate its effectiveness in bonding the top layer (PLA) and agar foil together for future iterations. As depicted in Figure 6.23, the BioTak adhesive successfully adhered the two materials, providing a strong connection between them. This finding suggests that the BioTak adhesive can be used to secure the PLA and agar foil layers in future iterations of the living label.

There is an interesting element to this design, which is that the colony appears to be more red. Further experimentation showed that the cause of this new aesthetic was attributed to the glycerol ingredient of the agar foil (see appendix 6.9). This red colour however, does not necessarily imply that the Flavobacteria cannot be used as a sensor anymore. To explore the communicative value of the Flavobacteria enclosed by agar foil, one of the samples was subjected to temperature changes by placing it in the fridge (approximately 4°C) and then returning it to room temperature. Figure 6.24 demonstrates that despite the variation in iridescent colour, the changes in temperature can still be observed as before. This suggests that the altered living aesthetic does not hinder the ability of the Flavobacteria to serve as a sensor for temperature fluctuations.

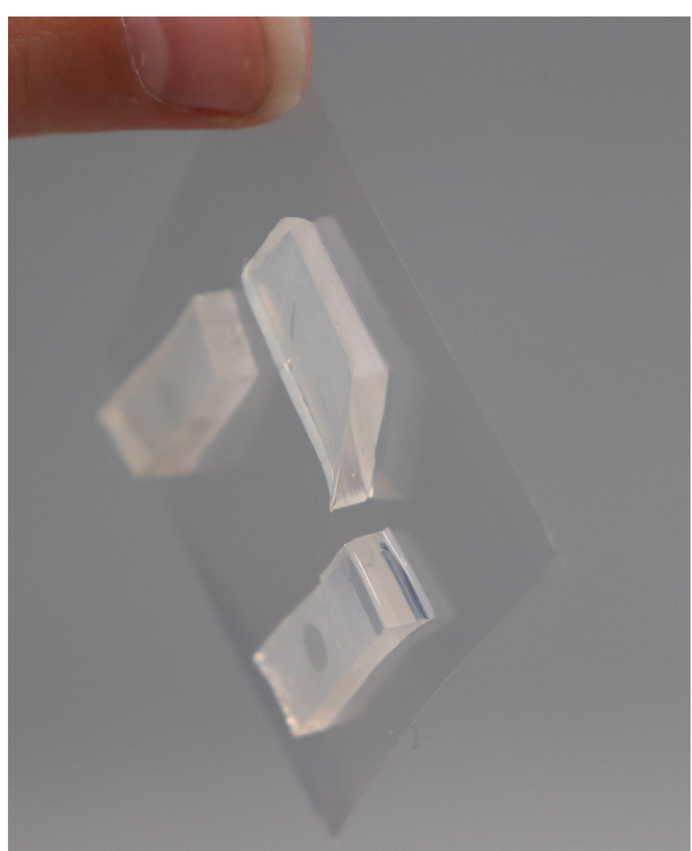
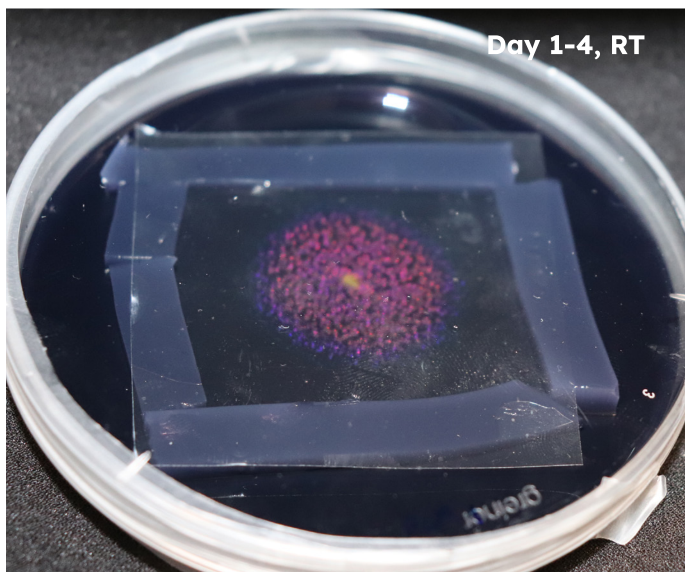
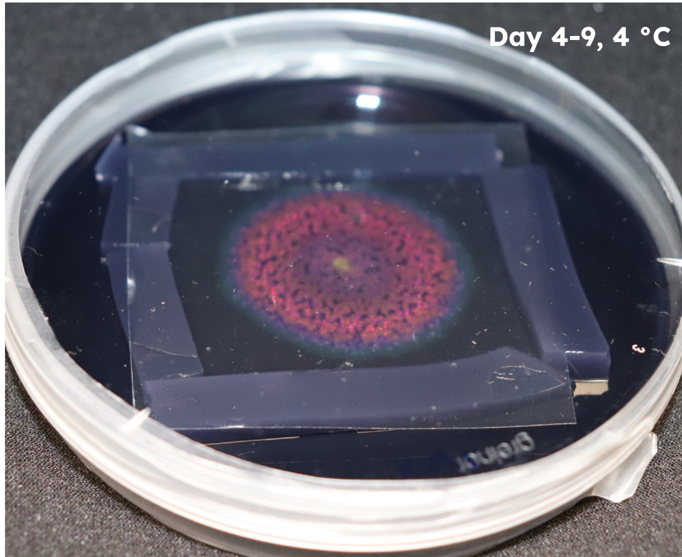


Figure 6.23- *Agar foil sticking to BioTak adhesive on PLA*





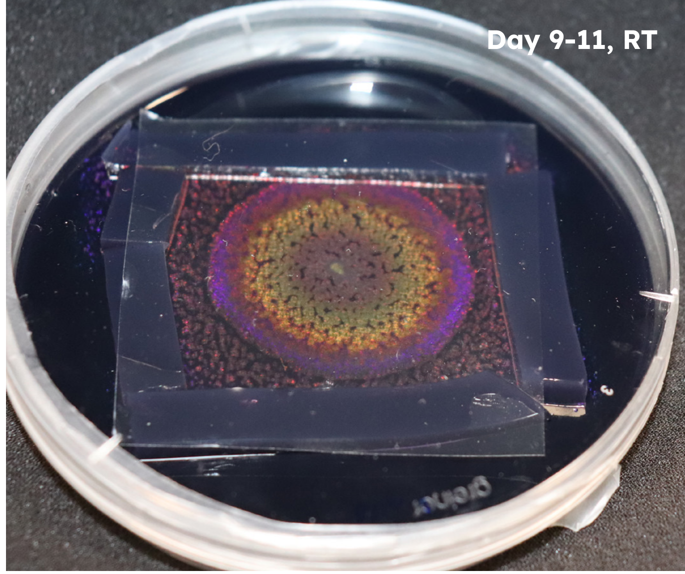
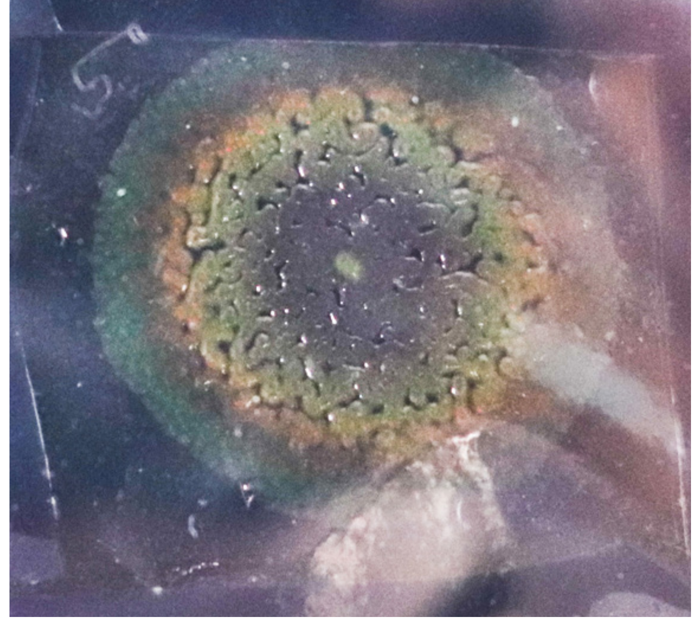


Figure 6.24- *C. lytica with headspace in alternated temperatures*

In addition to the iridescent colour and temperature rings, the texture of the Flavobacteria colony also provides valuable information. Different parts of the colony exhibit distinct textures, as illustrated in Figure 6.25. This indicates that the communicative value of the Flavobacteria colony is not limited to specific lighting conditions or viewing angles, as texture can be seen at any time.

Figures 6.26 and 6.27 demonstrate that the communicative value of the colony can be observed under various conditions and from different angles, with the texture of the colony contributing to its ability to convey information.



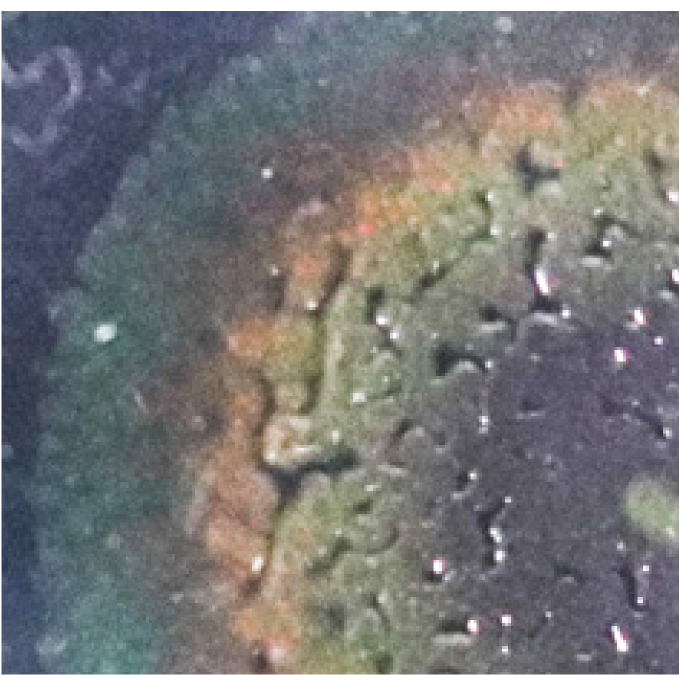
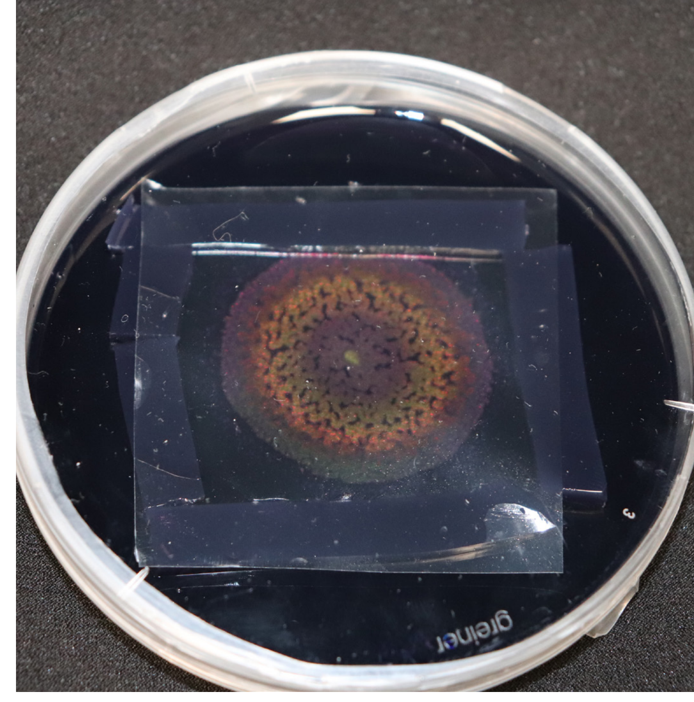
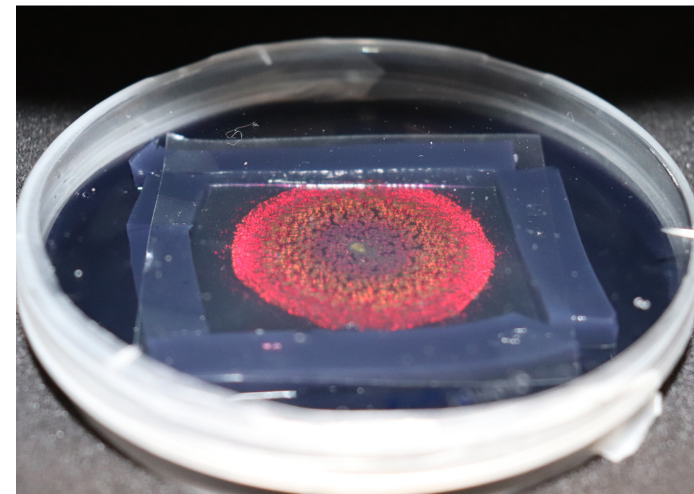


Figure 6.25- *Texture of C. lytica colony after undergoing temperature changes under headspace*





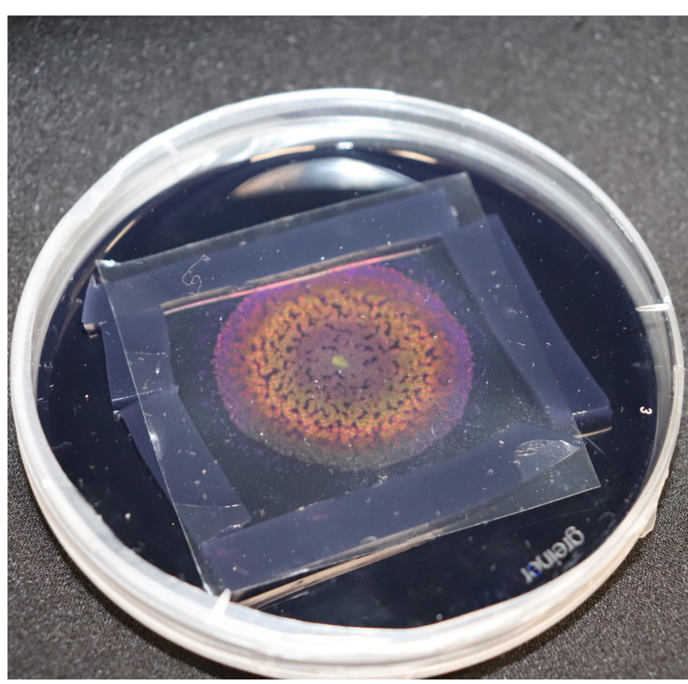
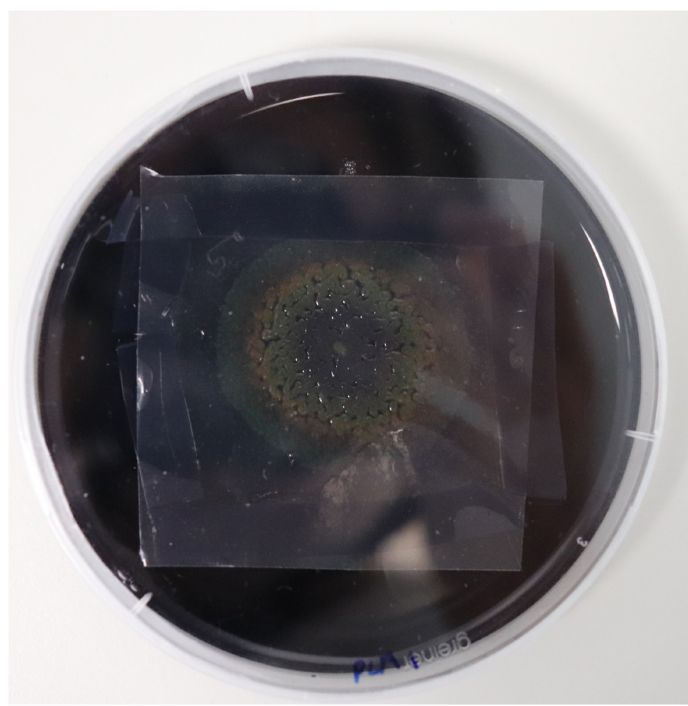
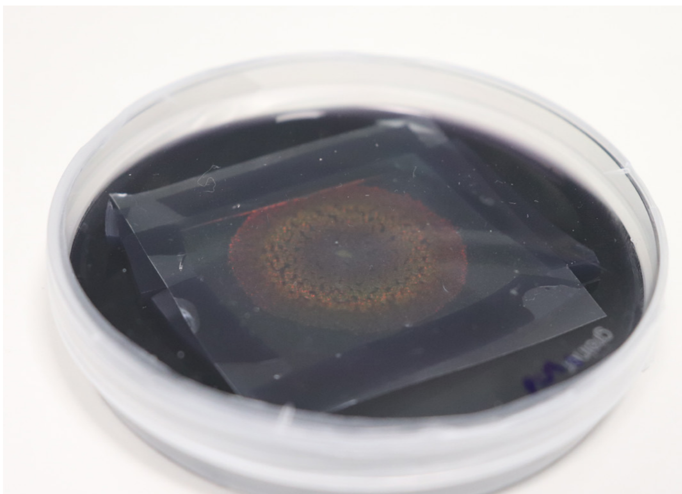


Figure 6.26- *C. lytica colony under headspace, captured from several viewing angles and illuminated with flash*





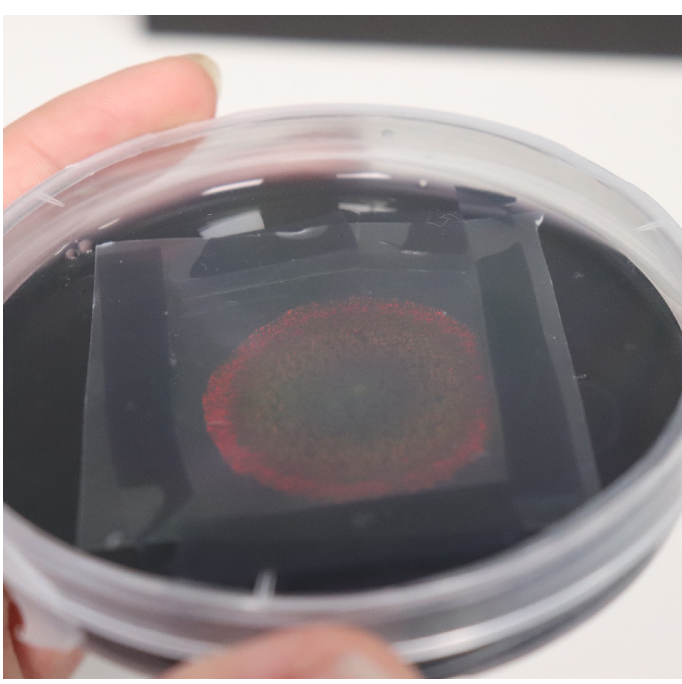
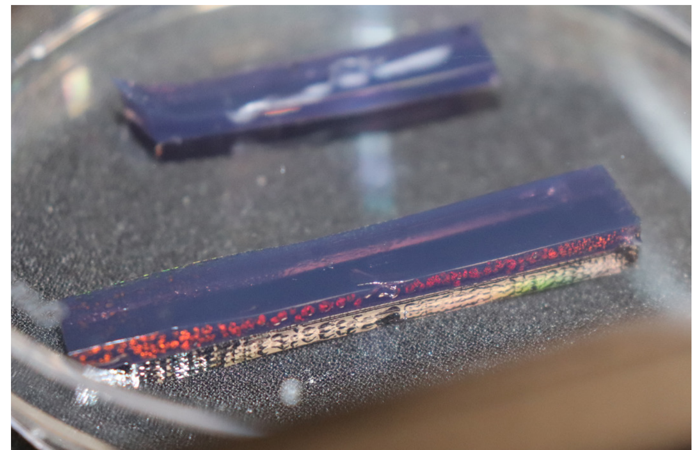


Figure 6.27 - C. lytica colony under headspace, captured from several viewing angles with no additional illumination

In conclusion, the combination of agar foil with the transparent PLA material from Bio4Life creates a suitable environment for Flavobacteria to generate structural colour and communicate temperature changes. The PLA material remains transparent and unaffected by temperature variations, making it well-suited for the purpose of the living label.

Furthermore, it was observed that Flavobacteria are able to grow on agar foil, even though it does not contain specific nutrients or salts. This may be attributed to the agar foil absorbing nutrients from the MAR medium, as indicated by the presence of the black pigment (Figure 6.28). This phenomenon could be investigated further for other parts of the label, for instance as an alternative medium.



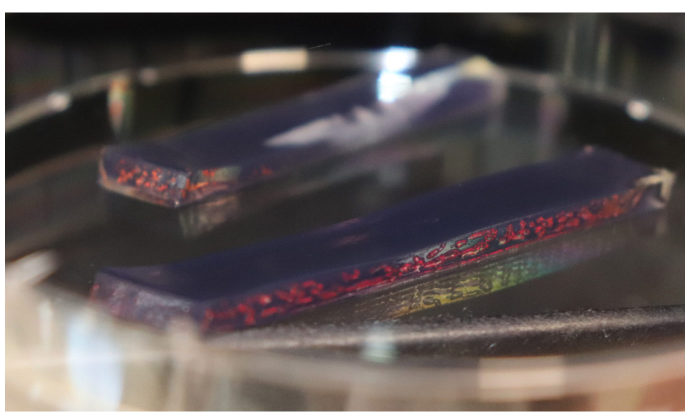


Figure 6.28 - C. lytica growing on agar foil extracted from the physical headspace

CH. 7

Activation Method

Introduction

In order to use the living label in a real life context, it is important that it can be preserved until the use phase. This because it is not desirable that the Flavobacteria start growing the moment the living label is assembled, but rather when it enters its use phase. This is why in this chapter, preservation methods of Flavobacteria were researched and a more detailed explanation of freeze drying is included.

Content

7.1 Flavobacteria Preservation **p. 64**

7.2 Freeze Drying Flavobacteria **p. 65-69**
7.2.1 Preparing the Samples
7.2.2 Consistancy after Freeze Drying
7.2.3 Rehydration

Method

Literature research & iterative experimentation

7.1 Flavobacteria Preservation

A preservation method for Flavobacteria commonly employed is freezing, which involves subjecting the Flavobacteria to ultra-low temperatures (-80°C). Addition of glycerol serves to protect bacteria from potential damage caused by formation of ice crystals. However, preserving the living label at such low temperatures is impractical due to specialised freezer requirements associated with this approach. Consequently, freezing was not deemed suitable for preservation of the living label.

Alternatively, freeze drying, also known as lyophilization, represents another widely adopted method for bacteria preservation. By subjecting the bacteria to a process of freeze drying, their moisture content is effectively eliminated, leaving them resilient to environmental conditions. Unlike freezing, freeze-dried bacteria do not necessitate specialised storage environments and solely require protection from moisture to maintain their viability. It is worth noting, however, that the freeze drying process itself can pose a potential risk to the bacteria, as they are exposed to harsh conditions that might damage them. To reduce the amount of non viable cells after freeze drying, cryoprotectants are used (Cui et al., 2018). These protectants prevent damage caused by freezing, similar to the glycerol that is added when freezing the Flavobacteria in the -80°C freezer.

Considering the specific requirements of the living label, freeze drying appears to be the most suitable preservation method for the Flavobacteria. This approach ensures their extended shelf life, allowing for their activation and subsequent functionality upon entering the use phase of the label.

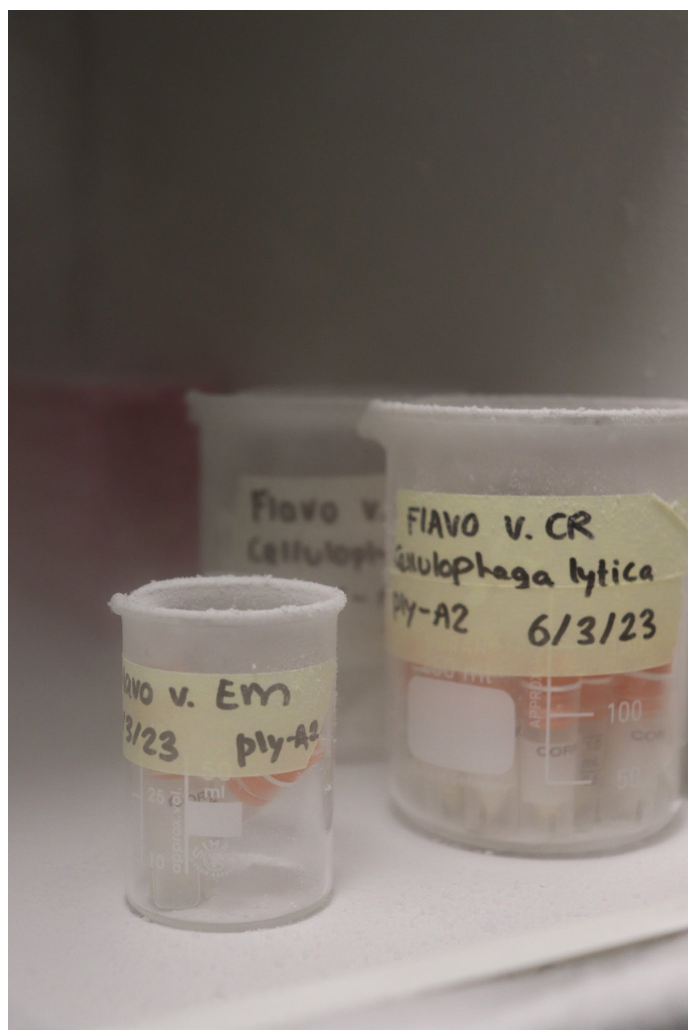


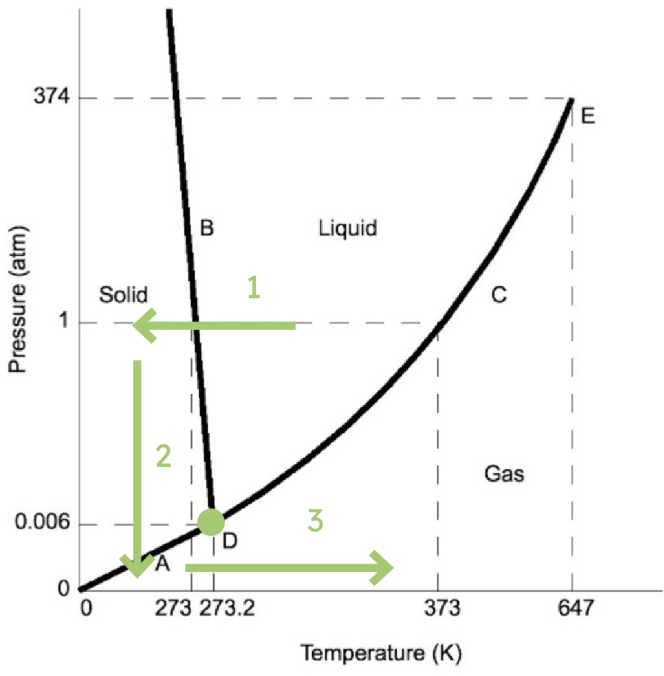
Figure 7.1 - *Flavobacteria in -80°C freezer*

Figure 7.2 - *Freeze-dried Flavobacteria*

7.2 Freeze Drying Flavobacteria

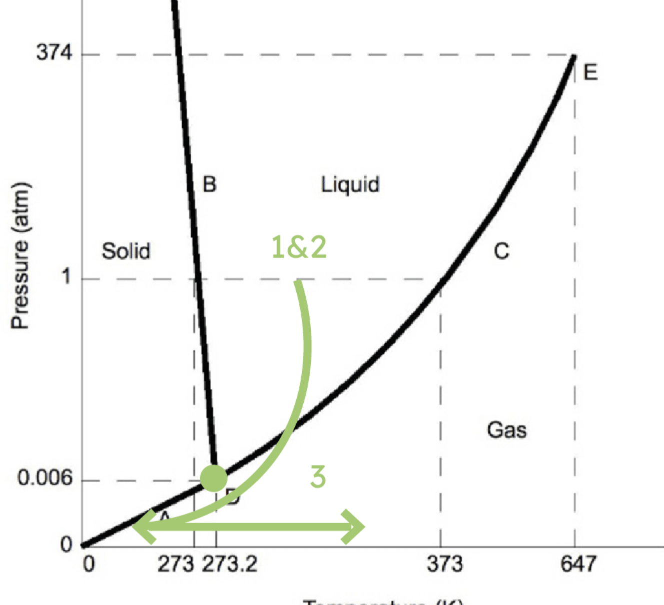
Freeze drying, also known as lyophilization, is based on the principle of sublimation, wherein a solid transitions directly into a gas without passing through the liquid phase (Shukla, 2011). The objective of freeze drying is to extract the majority of the water content from the material being processed. At the conclusion of the freeze drying process, the residual water content typically ranges between 1% and 4% (Shukla, 2011).

Figure 7.3a illustrates the phase diagram of water, indicating that during freeze drying, the sample is frozen below the triple point temperature of water to promote sublimation rather than melting. The freeze drying process can be divided into three main stages: freezing, primary drying (sublimation), and secondary drying (desorption). However, in practice, the freeze dryer used in the BioLab appears to combine the freezing and primary drying phases, denoted by numbers 1 and 2 in Figure 7.3b. Consequently, the sample swiftly enters the gas phase just prior to freezing, resulting in bubbling. To achieve the desired freeze-dried outcome, samples are initially frozen in the -80°C freezer for a minimum of one hour before being transferred to the BioLab's freeze dryer, thereby facilitating the intended freeze drying process outlined in Figure 7.3a.



- Triple point
- 1 Freezing
- 2 Sublimation
- 3 Desorption

Figure 7.3a *- Phase Diagram of water adapted from (Encrenaz, 2014)*



- Triple point
- 1 Freezing
- 2 Sublimation
- 3 Desorption

Figure 7.3b *- Phase Diagram of water adapted from (Encrenaz, 2014)*

7.1.1 Preparing the Samples

Prior to subjecting the Flavobacteria to the freeze drying process, they must be harvested from the MAR medium plates (Figure 7.4) and subsequently prepared in a solution containing a suitable protectant (Figure 7.5). In this project, various protectants were investigated, including:

10% sucrose
10% sucrose + liquid medium
10% milk
10% milk + liquid medium

These protectants can be seen in Figure 7.6. The complete procedure for preparing the Flavobacteria samples can be found in Appendix 7.3. This protocol was predominantly derived from the bacterial freeze drying protocol provided by OPS Diagnostics (Bacterial Freeze Drying Protocol, n.d.).

7.1.2 Consistency after Freeze Drying

The usage of various protectants for the Flavobacteria during freeze drying resulted in different consistencies after the process. Apart from the use of different protectants, variations were also made in freeze-drying time (24 hours or 48 hours) and pre-freezing samples before the freeze drying process or not (Figure 7.7). It is anticipated that freeze drying will result in a powdery consistency due to the extraction of a significant portion of the water from the solution. Failure to achieve this powdery consistency raises concerns regarding the success of the freeze drying process.

In Figure 7.7, it can be observed that the solutions that underwent pre-freezing prior to entering the freeze dryer exhibit a more pronounced powder-like substance. Among these pre-frozen solutions, the protectants containing sucrose and milk demonstrate the most favourable consistency outcomes.

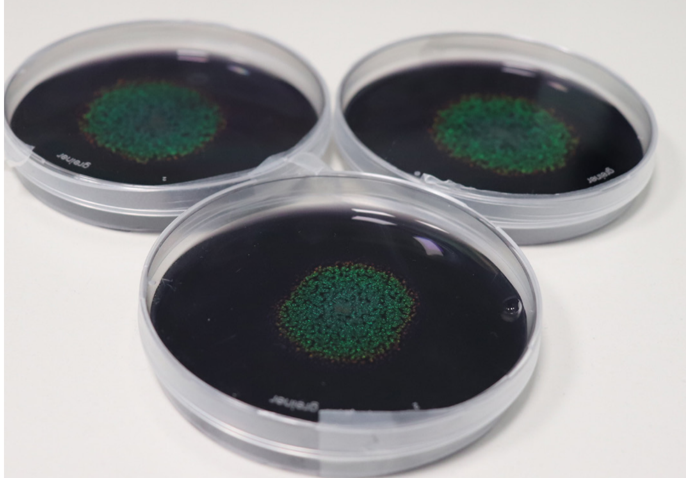


Figure 7.4 - Petri dishes with Flavobacteria ready for harvest

Figure 7.5 - *C. lytica in solution with protectants*

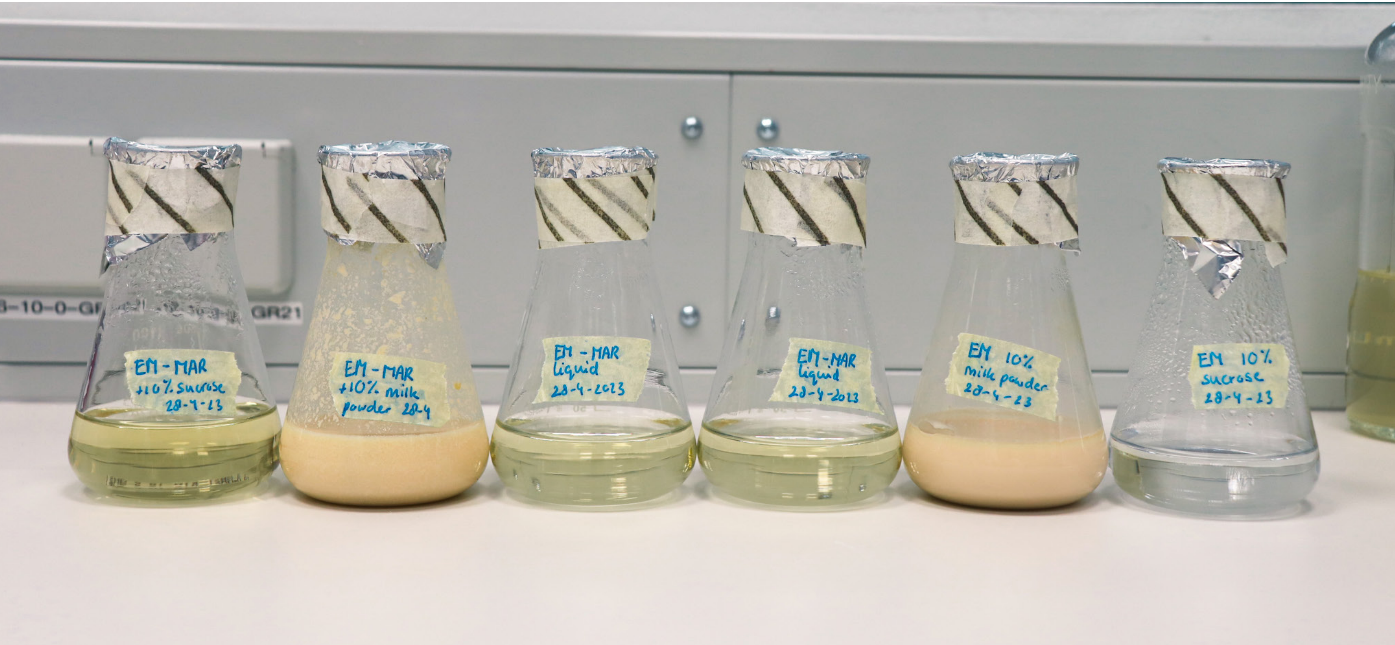


Figure 7.6- *Cryoprotectants involved in the experiment*

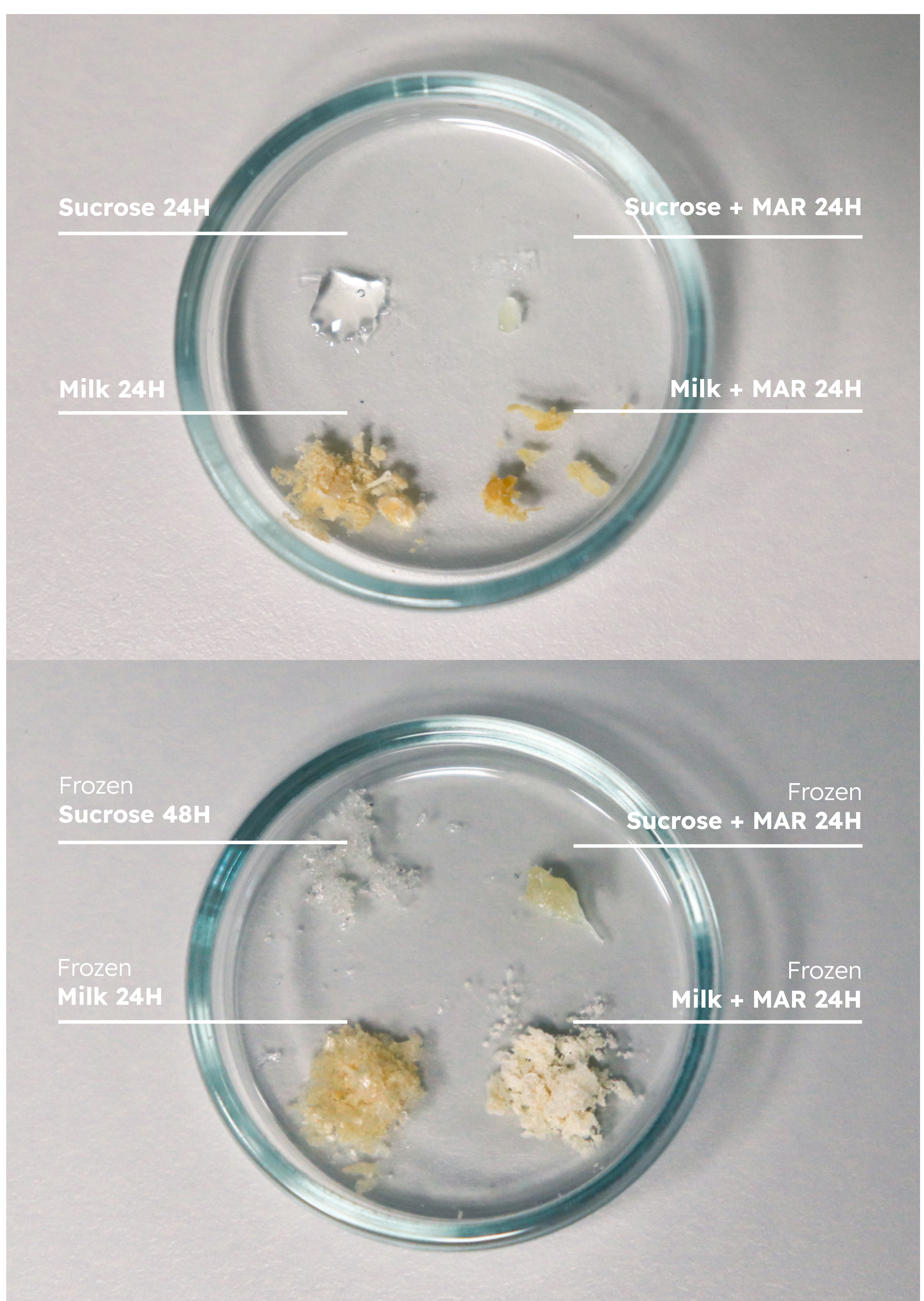


Figure 7.7- *Consistency of freeze-dried C. lytica with variations in protectants, freeze-drying time and pre-freezing*

7.1.3 Rehydration

The rehydration process involved transferring the freeze-dried Flavobacteria onto a standard MAR medium petri dish. While the addition of NaCl (salt) at a concentration of 0.9% has been suggested to enhance rehydration (Bacterial Freeze Drying Protocol, n.d.), it is expected that the MAR medium alone, which contains approximately 3% salt concentration, is sufficient for rehydrating the Flavobacteria.

As mentioned previously, four different protectants were tested, with variations in pre-freezing or not. The time of freeze drying was also varied with the sucrose protectant to achieve the desirable consistency. As shown in Figures 7.9 to 7.16, all protectants effectively safeguarded the Flavobacteria during the freeze drying process, as evidenced by their growth and the presence of structural colour after rehydration. There does not seem to be a difference in the growth rate of the Flavobacteria with different protectants, compared to the control sample (Figure 7.8).

Notably, the choice of protectant does influence the visual appearance of the colony. The milk and milk + liquid medium protectant appears to create a veil-like layer over the colony, which slightly disrupts the structural colour (Figures 7.9 to 7.12). Conversely, the sucrose and sucrose + liquid medium protectant does not exhibit this effect and allows the Flavobacteria to grow in their typical manner (Figure 7.13 to 7.16). The fact that the frozen sucrose protectant was freeze-dried for 48 hours instead of 24, does not seem to impact the growth or visual appearance compared to the other protectants with sucrose. The full documentation of this experiment can be found in Appendix 7.1 and 7.2.

In order to maintain the desired structural colour of Flavobacteria for the living label, it is crucial to avoid any unwanted disruption. As observed, the use of milk protectants introduces a veil-like layer that interferes with the aesthetics of structural colour. Therefore, milk protectants are not suitable for this purpose. On the other hand, both sucrose protectants do not disturb the aesthetics of the Flavobacteria colony after rehydration.

As illustrated in the previous subchapter, there is a significant difference in consistency between the sucrose protectant with liquid medium and the sucrose protectant without liquid medium after the freeze drying process. Notably, the sucrose protectant with liquid medium does not achieve a powder-like consistency, indicating that it may not have undergone complete freeze drying.

Considering these observations, the sucrose protectant without liquid medium is most suitable for the freeze drying process of Flavobacteria in the context of the living label. This choice ensures that the Flavobacteria achieve a powder-like consistency, indicative of successful freeze drying, while also preserving the desired structural colour after rehydration.

Figure 7.8 - *C. lytica control sample, day 6*

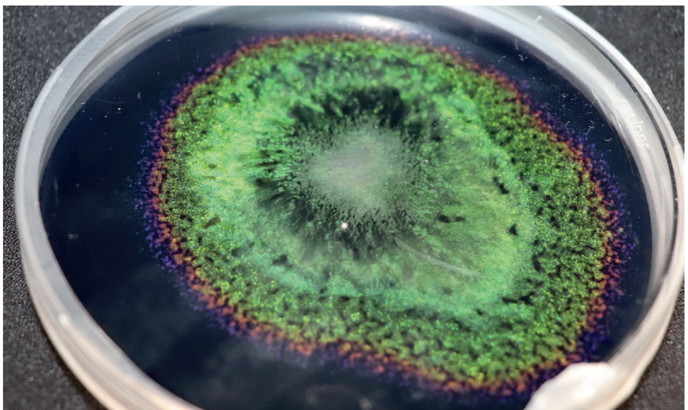


Figure 7.9 - *Milk, 24H, day 6*

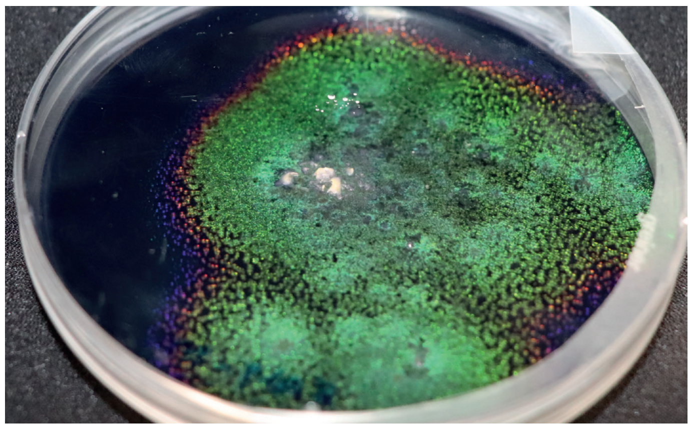


Figure 7.10 - *Milk+ liq medium, 24H, day 6*

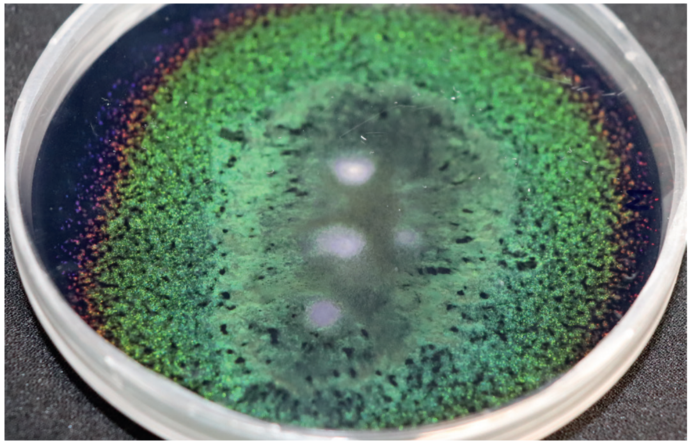


Figure 7.11 - *frozen Milk, 24H, day 7*

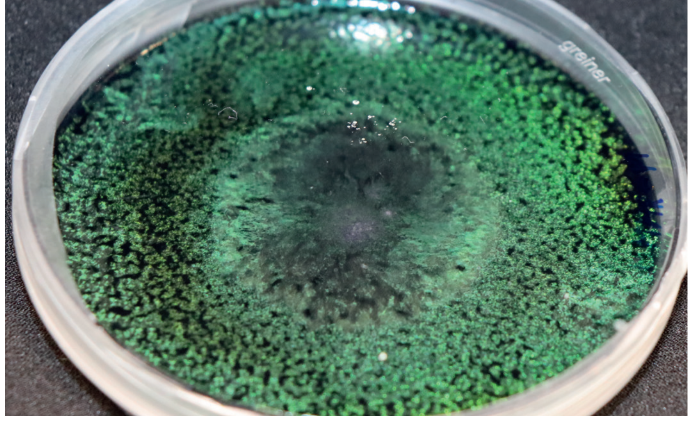


Figure 7.12 - *frozen Milk+ liq medium, 24H, day 7*

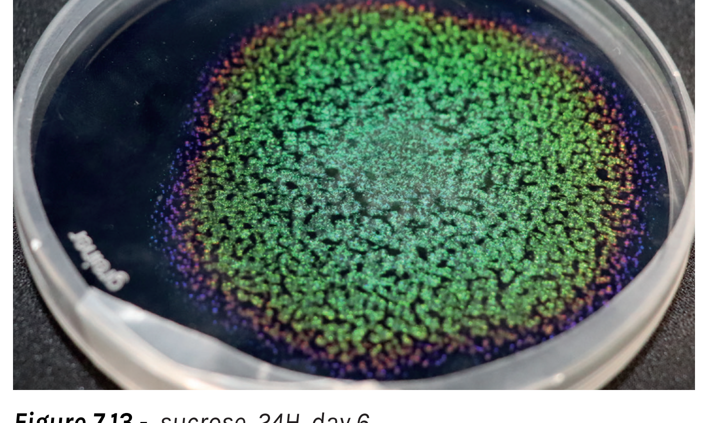


Figure 7.13 - *sucrose, 24H, day 6*

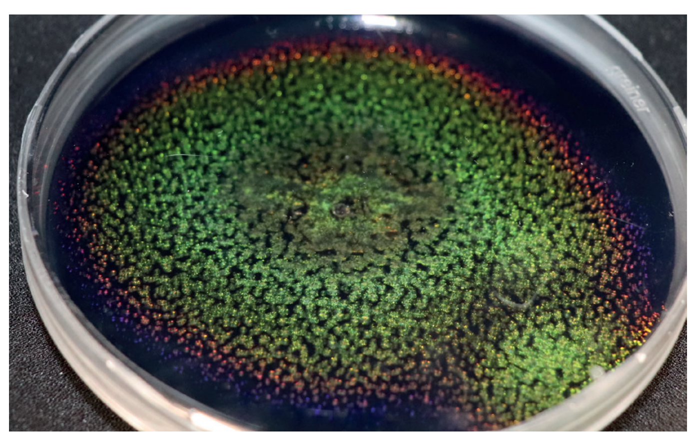


Figure 7.14 - *sucrose+ liq medium, 24H, day 6*

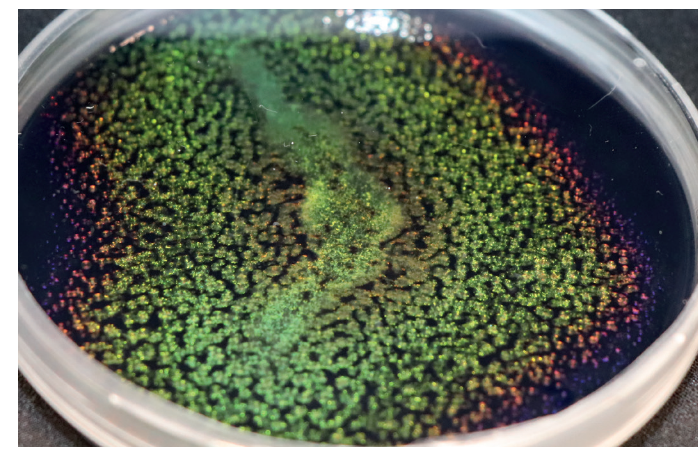


Figure 7.15 - *frozen sucrose, 48H, day 6*

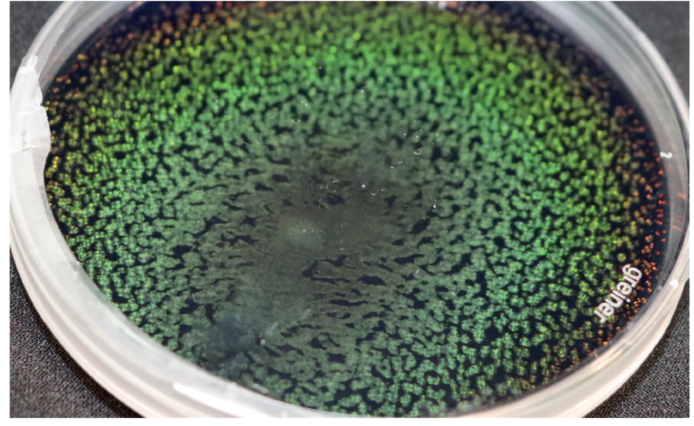


Figure 7.16 - *frozen sucrose+ liq medium, 24H, day 7*

CH. 8

Alternative Medium

Introduction

The MAR medium, which is mostly used for growing Flavobacteria, exhibits certain disadvantages when considering its application in the living label context. These drawbacks create the need to explore alternative mediums that address the specific requirements of the project. Therefore, in this chapter, alternative mediums for Flavobacteria in the living label will be explored.

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8.2 Inital Explorations p. 73

8.3 Freeze Drying MAR Medium p. 74-76

Method

Iterative experimentation

8.1 MAR Medium

To understand the specific direction in which an alternative medium needs to be found, the disadvantages of MAR medium are first discussed.

Cracking
One significant issue with the MAR medium is its tendency to crack when bent (Figure 8.1). This fragility becomes problematic when incorporating the medium into flexible materials. Therefore, it is crucial to either find a method to enhance the flexibility of the medium or identify a new, more flexible medium altogether.

Hydration
Another challenge arises from the need to keep the freeze-dried Flavobacteria away from moisture until they are ready for activation. Since the MAR medium itself is already hydrated, it makes it difficult to prevent moisture exposure during storage. This presents a problem in maintaining the viability of the freeze-dried Flavobacteria until their intended use.

Gelling Agent
Furthermore, the MAR medium is primarily available in a gel form, as it utilises agar as a gelling agent. This means that the medium can only be in liquid form at higher temperatures. Since a solution against hydrating the freeze-dried Flavobacteria prematurely would be to hydrate the medium just before the use phase, the high temperature would complicate the practicality of preparing the medium at that stage. Moreover, exposing the Flavobacteria to these higher temperatures could potentially harm their viability, adding another layer of complexity to the process.

Due to these undesirable aspects, this chapter explores alternative mediums that address these limitations and align more effectively with the requirements of the project.

Figure 8.1 - *Cracking behaviour of MAR medium*

8.2 Inital Explorations

In the pursuit of finding an alternative to the MAR medium for the living label, various options were explored, including different hydrogels such as Calcium Alginate and freeze-dried Calcium Alginate, as well as other materials like the water soluble Polyvinylalcohol (Figures 8.2 to 8.9). However, these alternatives exhibited drawbacks that affected the growth, but also the structural colour of Flavobacteria causing it to lose its communicative value. A comprehensive account of these experiments can be found in Appendix 8.1 to 8.5.

One significant drawback arising from the exploration of alternative mediums is the lack of existing characterization of the living aesthetics of Flavobacteria on these new mediums. This poses a challenge in maintaining the communicative value of Flavobacteria, as the interaction between the medium and the bacteria may become increasingly complex and unpredictable. As a result, the regular MAR medium was reconsidered as a potential solution. In the next subchapter, experiments will be discussed that focus on preserving the MAR medium and mitigating its cracking behaviour through the process of freeze drying.

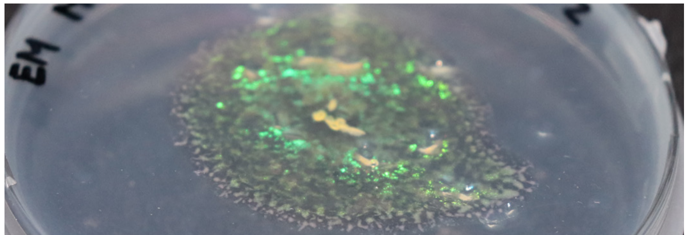


Figure 8.2- *C. lytica colony on MAR medium without nigrosine*

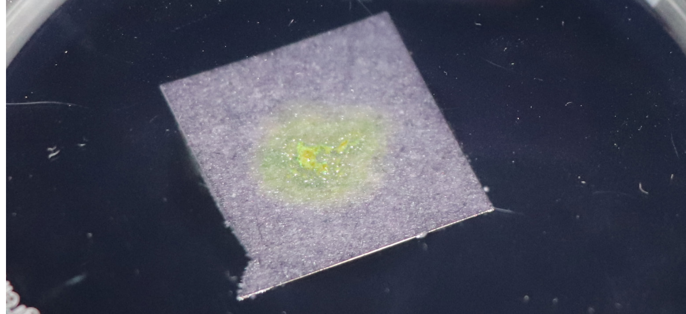


Figure 8.3 - *C. lytica colony on PaperWise from Bio4Life*

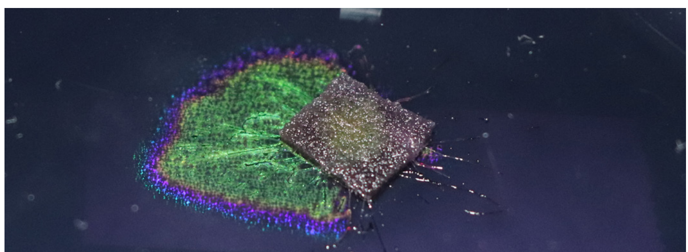


Figure 8.4 - *C. lytica colony on Polyvinylalcohol (PVA)*

Figure 8.5 - *C. lytica on calcium alginate*

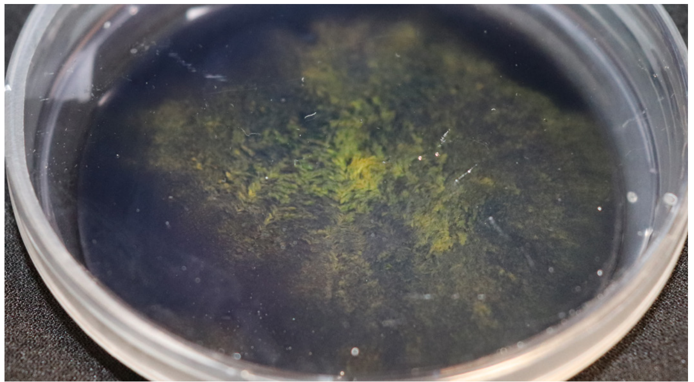


Figure 8.6 - *C. lytica on calcium alginate*

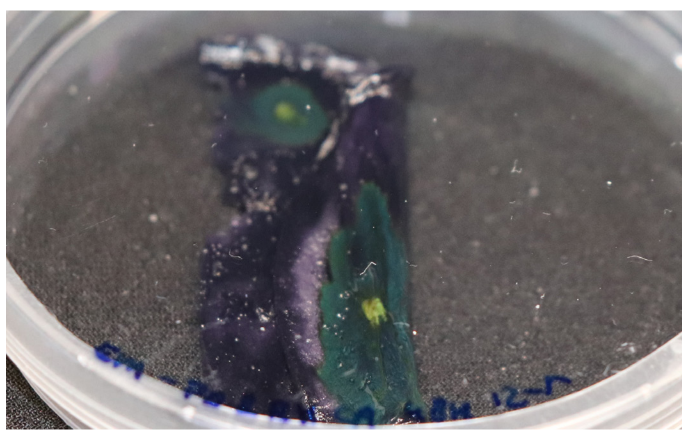


Figure 8.7 - *C. lytica on freeze-dried and rehydrated calcium alginate*

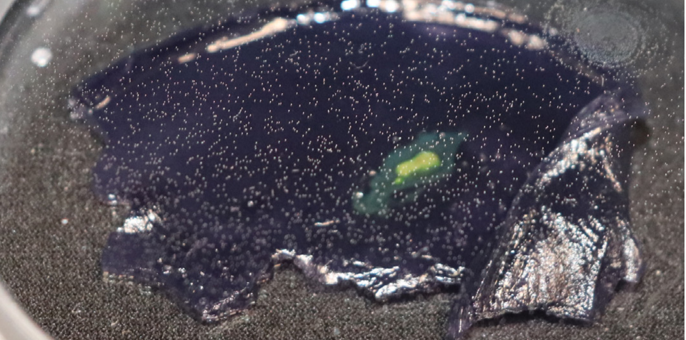


Figure 8.9 - *C. lytica on freeze-dried and rehydrated calcium alginate*

8.3 Freeze Drying MAR Medium

By subjecting a material to freeze drying, long-term storage can be enabled. Therefore, freeze drying was used to preserve the MAR medium. By freeze drying the MAR medium, combining it with freeze dried Flavobacteria no longer poses a problem since the medium will be dehydrated. Additionally, since the physical properties of this new freeze-dried medium might differ from the regular MAR medium, improvements might be made in the strength of the material, avoiding cracking. This chapter further explains the process of freeze drying the MAR medium.

Freeze drying process
The freeze drying process involved placing the medium in a standard Petri dish and securing the lid with two pieces of tape to prevent it from coming off during the process (Figures 8.10 and 8.11). It was important not to pre-freeze the medium before freeze drying, as this would result in a rough texture (Figure 8.12). The freeze drying of the MAR medium required a duration of 24 hours. During the freeze drying process, the height of the medium significantly decreased (Figure 8.13). Three different heights of MAR medium were subjected to the freeze-drying process. The thinner the medium before the process, the more crumbled it looked after freeze drying (Figure 8.14).

Rehydration
After the freeze-dried MAR medium undergoes rehydration, its height is observed to be approximately 30-50% of its original height. This differs from the samples that were frozen before freeze drying, which exhibit a height of around 75% of the original height. The rehydration process requires approximately 40-50% of the original volume in demi water. However, when the medium is frozen before freeze drying, a larger volume of approximately 60% of the original volume is needed for rehydration. Detailed data supporting these percentages can be found in Appendix 8.8.

Regarding the consistency of the rehydrated MAR medium, notable differences are observed among the samples (Figures 8.15 to 8.17). The specific factors contributing to these differences are yet to be determined. In terms of flexibility, it is worth noting that the thinner samples show improvements compared to the original MAR medium.

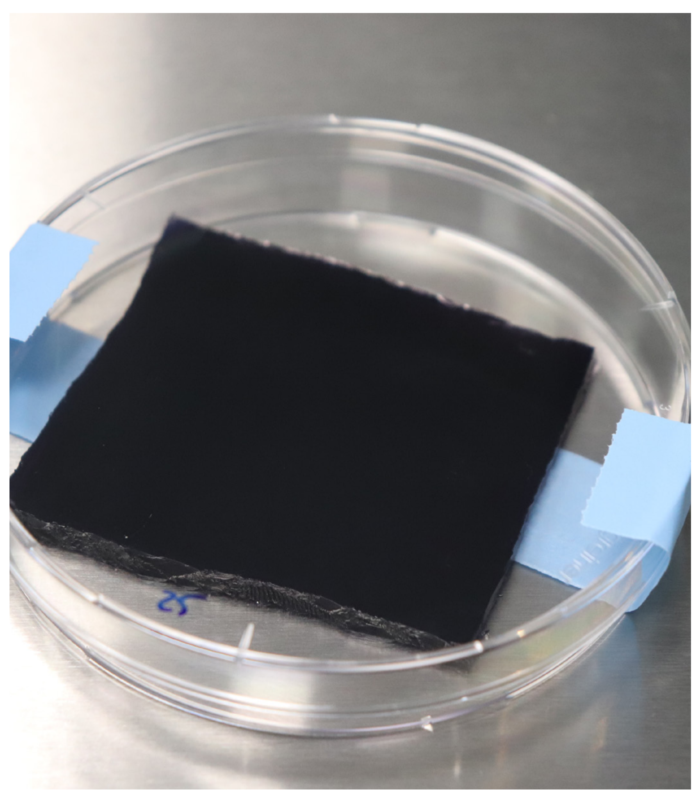


Figure 8.10 *- MAR medium before freeze-drying*

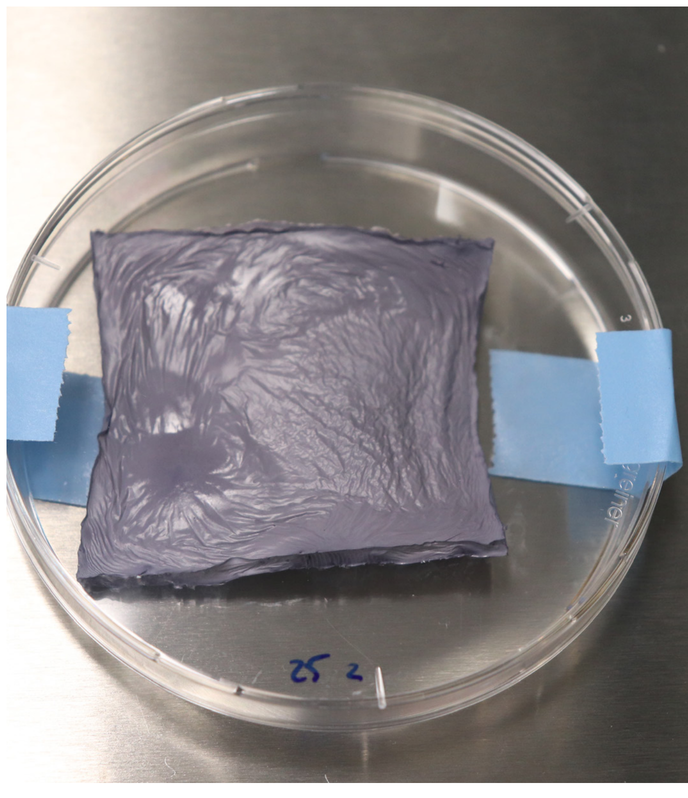


Figure 8.11 *- MAR medium after freeze-drying*

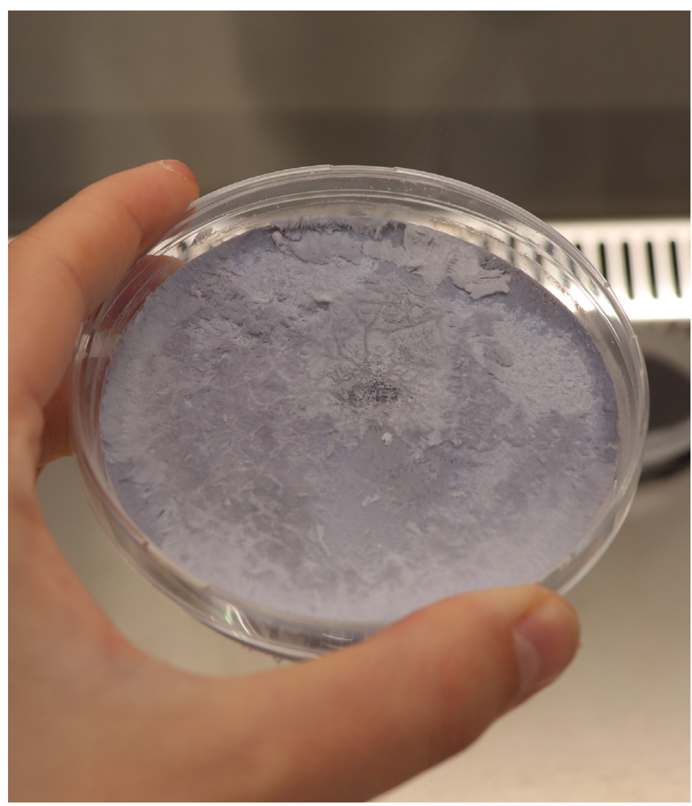


Figure 8.12- *Freeze dried MAR medium with Flavobacteria in sucrose protectant (pre-frozen)*

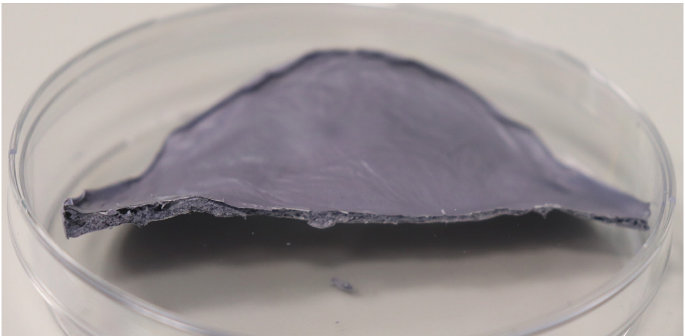


Figure 8.13 - *MAR medium after freeze-drying (original volume before freeze-drying 25 mL)*

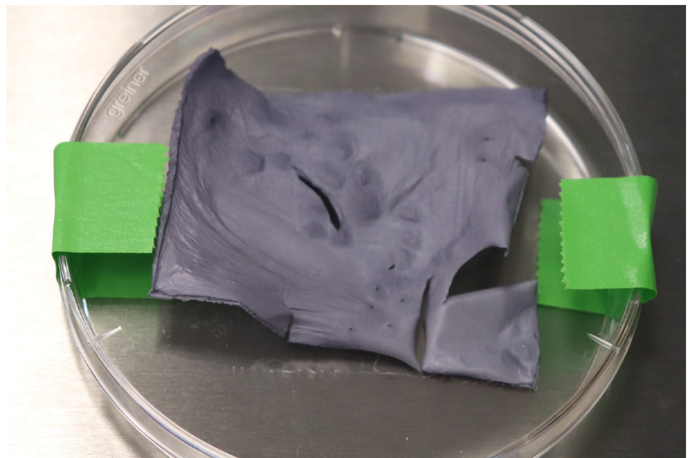


Figure 8.14 - *MAR medium after freeze drying (original volume before freeze-drying 10 mL)*

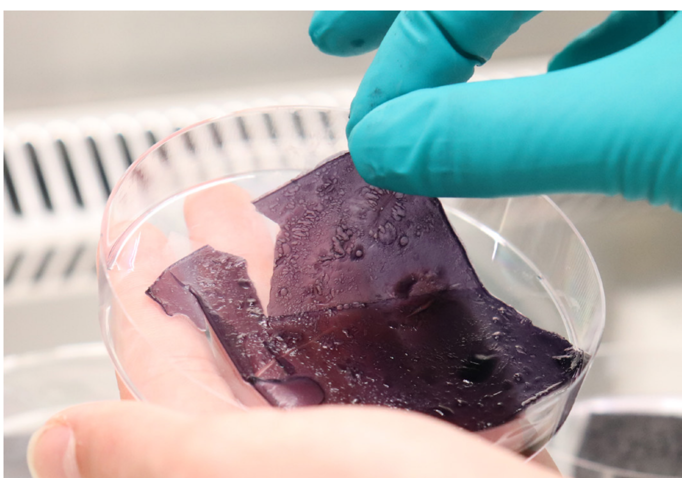


Figure 8.15 - *Rehydated MAR medium (original volume before freeze-drying 10 mL)*

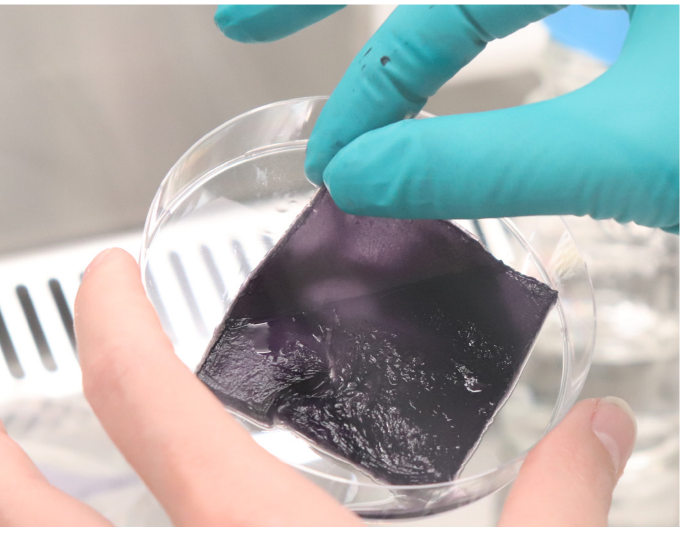


Figure 8.16 - *Rehydated MAR medium (original volume before freeze-drying 20 mL)*

Figure 8.17 - *Rehydated MAR medium (original volume before freeze-drying 25 mL)*

Inoculation
The Flavobacteria demonstrate successful growth on rehydrated MAR medium and retain their ability to produce structural colour (Figures 8.18 to 8.20). However, it is noteworthy that the surface texture of the rehydrated agar significantly influences the direction of Flavobacteria growth and the appearance of the structural colour. The presence of grooves or indentations on the surface seems to attract the bacteria, leading to their accumulation in those areas (Figure 8.18).

In conclusion, freeze drying the MAR medium offers the advantage of activating the medium at the use phase of the living label while providing a long shelf life. Furthermore, the rehydrated MAR medium exhibits sufficient flexibility for application in a flexible habitat such as a label. Additionally, Flavobacteria demonstrate successful growth and structural colour on this medium. However, further investigation is required to determine whether the communicative value of the structural colour remains intact when undergoing temperature fluctuations.

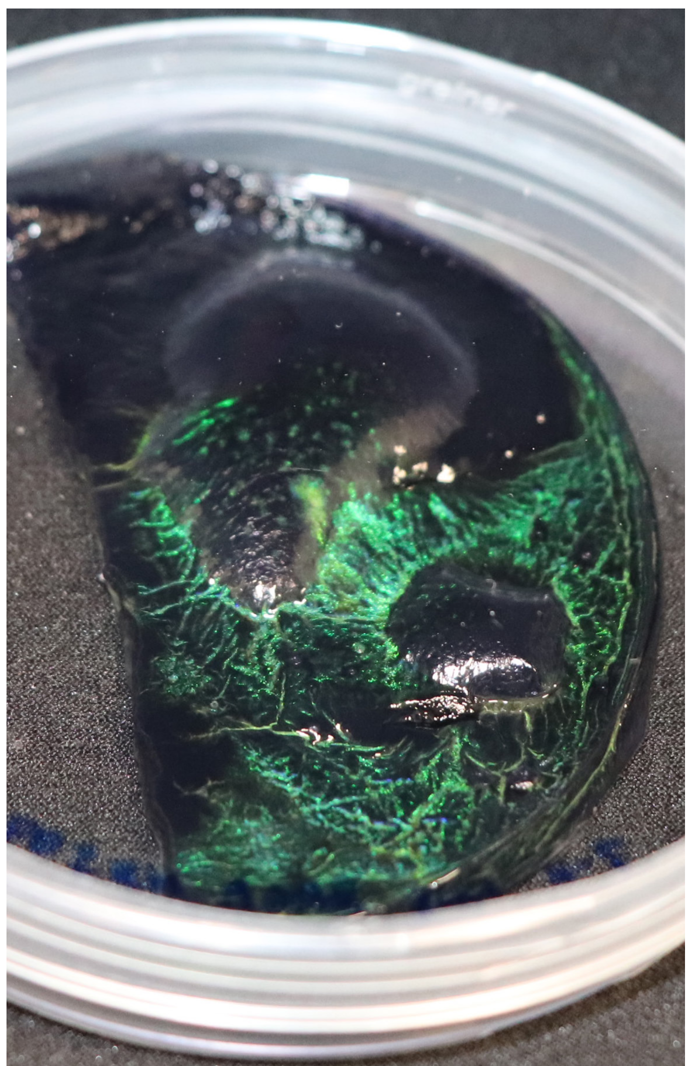


Figure 8.19 - *C. lytica growing on rehydrated MAR medium*

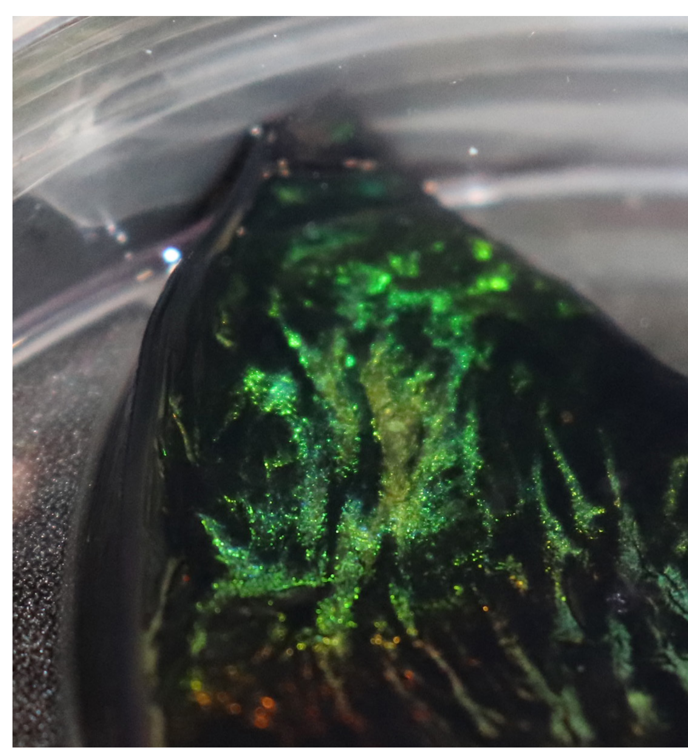


Figure 8.18 - *C. lytica growing on rehydrated MAR medium*

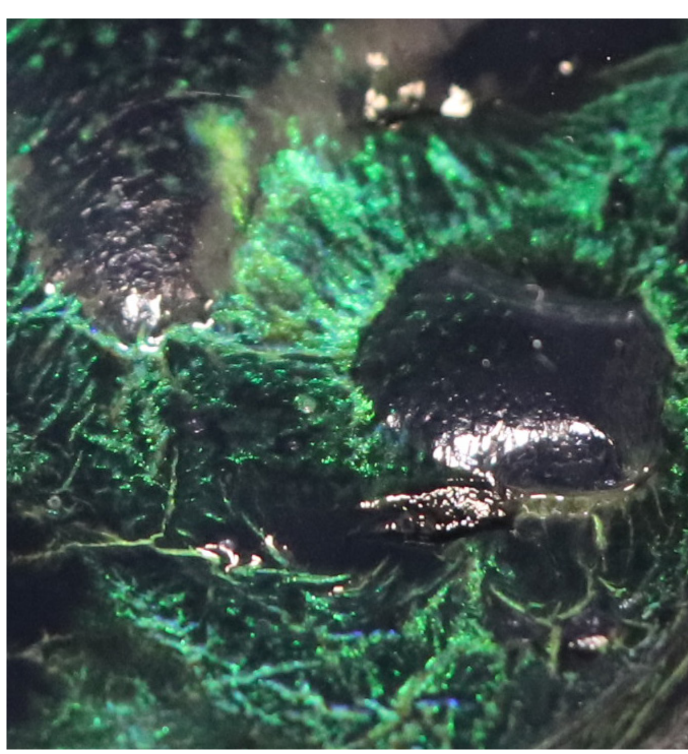
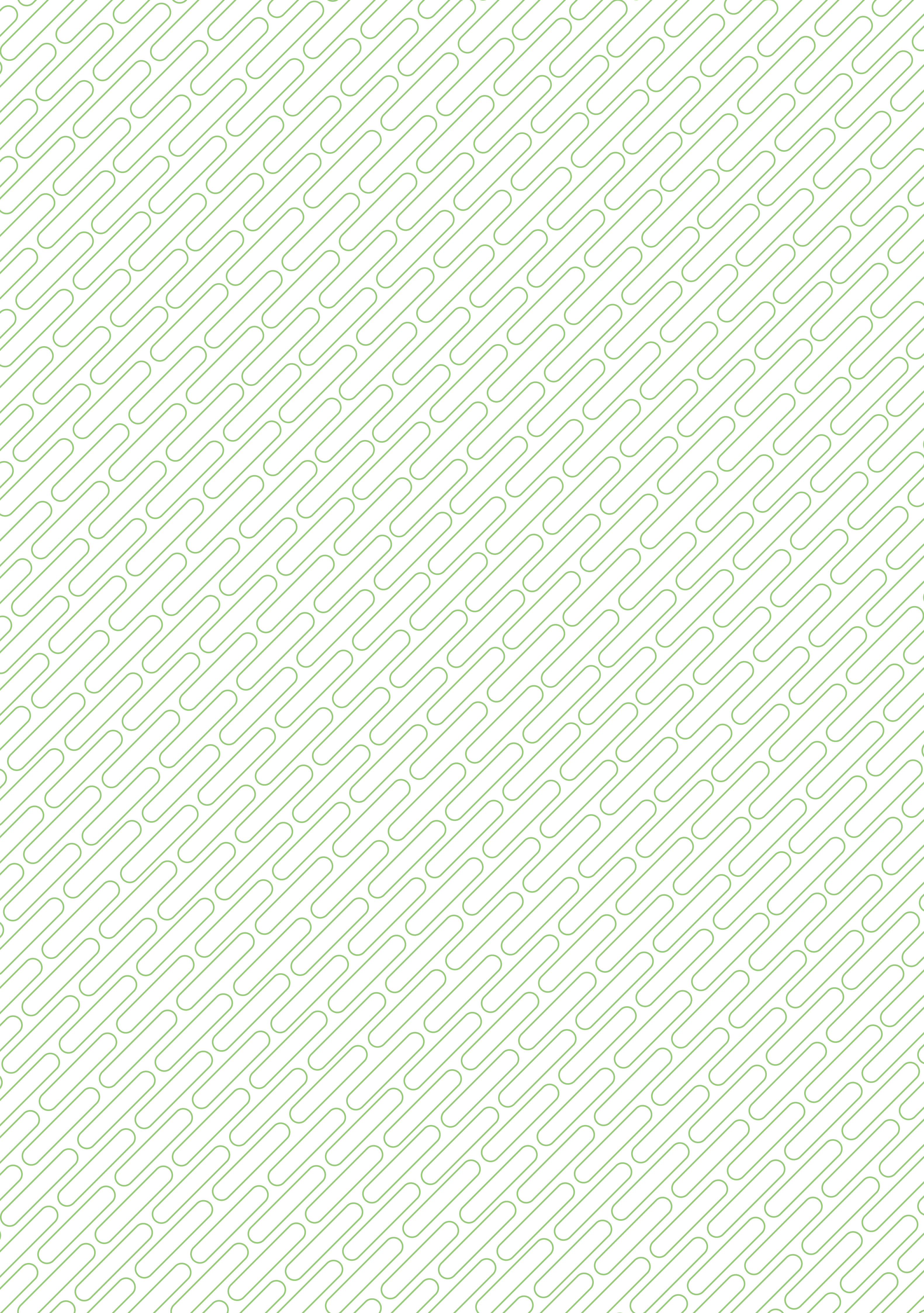
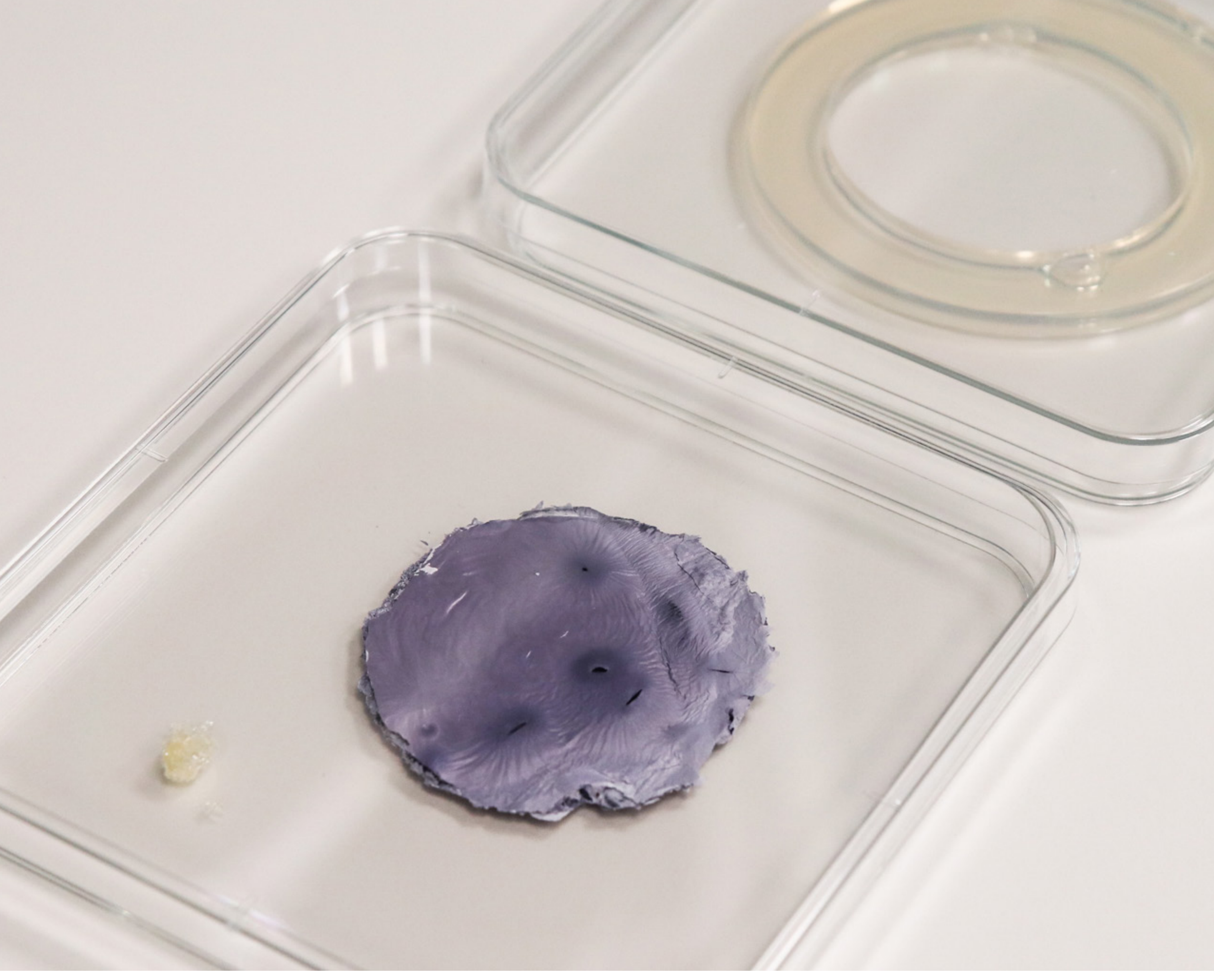


Figure 8.20 - *C. lytica growing on rehydrated MAR medium (close-up)*





CH. 9

Living Label

Introduction

In the previous chapters, the three different parts of the living label have been addressed, resulting in distinct outcomes that will be integrated in this chapter. Furthermore, a future product vison will be presented and guidelines for designers to work with Flavobacteria will also be incorporated.

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9.1 Living Label p. 80-84
9.1.1 Assembly Method
9.1.2 Rehydration/ Activation
9.1.3 Sensor Validation

9.1 Future Product Vision p. 85

9.1 Design Guidelines p. 86-87

Method

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9.1 Living Label

The envisioned label consists of various components, as depicted in Figure 9.1. Each component was a result of the exploration done in each chapter. The conclusion for each component can be found below. Additionally, the integration of the freeze-dried components of the label will be discussed.

Top Layer (Chapter 6): *The selection of a material to cover C. lytica while still enabling them to produce structural colour.*

The solution for the top layer involves creating a headspace for the Flavobacteria. This is achieved by placing a circular piece of PLA material from Bio4Life, adhered with BioTak adhesive, on a ring of agar foil. The height of the agar foil should exceed that of the medium to ensure the presence of a headspace.

Activation Method (Chapter 7): *Developing a mechanism to initiate the growth of C. lytica.*

Based on successful rehydration results, the chosen approach for activating the growth of C. lytica involves using freeze-dried Flavobacteria with a sucrose protectant. This dry powder can be placed on top of the medium and the growth process can be initiated in the use phase. This can be done either by externally adding water to the label or by encapsulating water in the label itself which can be opened/broken at the use phase to start rehydration.

Alternative Medium (Chapter 8): *Identifying a medium more suitable for the living label than the standard MAR medium.*

The preferred medium for the living label is freeze-dried MAR medium. This alternative offers the advantage of extended shelf life due to the dehydration of the medium. Additionally, it prevents premature activation of the freeze-dried Flavobacteria. The rehydrated freeze-dried MAR medium exhibits improved flexibility compared to regular MAR medium, making it more suitable for a flexible label. The structural colour formed by C. lytica on this medium does differ from the typical structural colour seen on regular MAR medium in standard conditions. However, as long as relative change can be seen in the living aesthetics of the colony with temperature fluctuations, it can still be used as a sensor.

Integration of Freeze-Dried Components

The initial integration of the living label involved combining two freeze-dried components: the freeze-dried Flavobacteria in sucrose protectant and the freeze-dried MAR medium. The objective was to assess the feasibility of activating both components simultaneously, replicating the conditions that would occur in the living label (see Figure 9.2). By bringing these freeze-dried elements together and introducing demi water, the activation process could be observed and evaluated. The outcomes of this integration provide insights into the compatibility and functionality of the freeze-dried components when combined, ultimately informing the further development of the living label.

PLA with BioTak adhesive
(Bio4Life)

Freeze Dried *Cellulophaga lytica* in sucrose

Agar Foil
(Agar, Glycerine and Water)

Freeze Dried MAR Medium

Figure 9.1 - *Components of the envisioned living label*

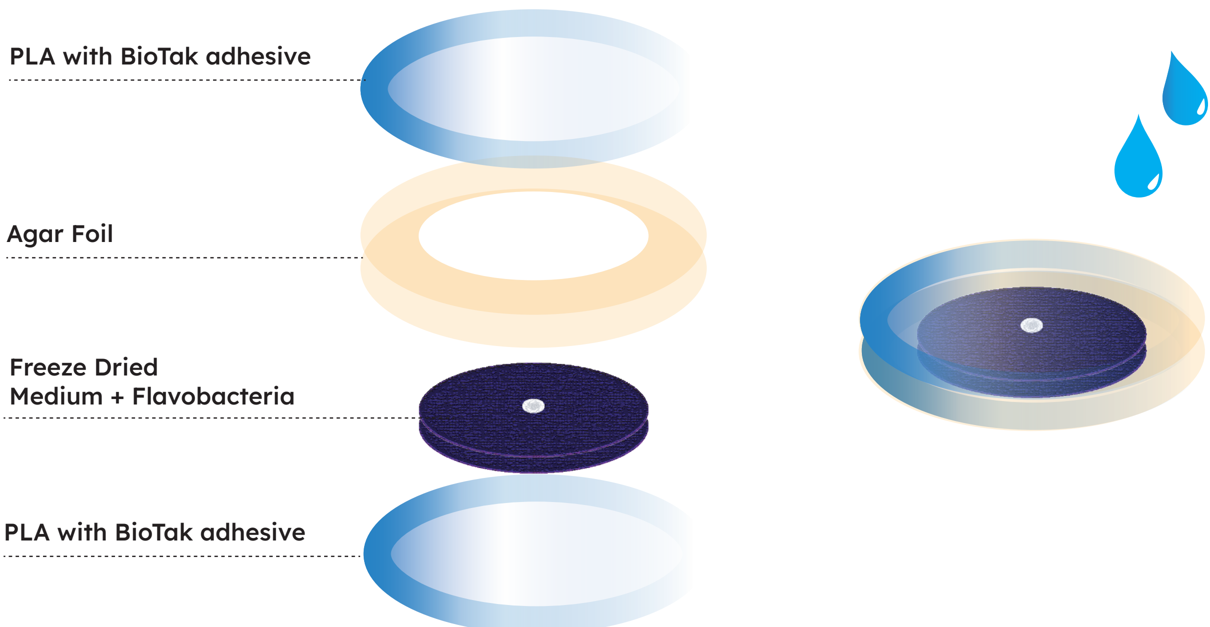


Figure 9.2 - *Exploded view of the envisioned label*

9.1.1 Assembly Method

To optimise the integration of Flavobacteria and MAR medium, a separate freeze-drying process was conducted for each component. This approach was adopted to address previous challenges encountered when freeze-drying them together, which resulted in undesirable surface texture and uneven distribution of Flavobacteria (refer to Appendix 9.1 for detailed results).

Figures 9.3 and 9.4 illustrate the separate freeze-dried components: the Flavobacteria and the MAR medium. Various thicknesses of freeze-dried MAR medium were tested during the experimentation to assess their impact on the final outcome after rehydration. Figure 9.5 shows the two different components after assembly.

Figure 9.3 *- Freeze-dried Flavobacteria in sucrose*

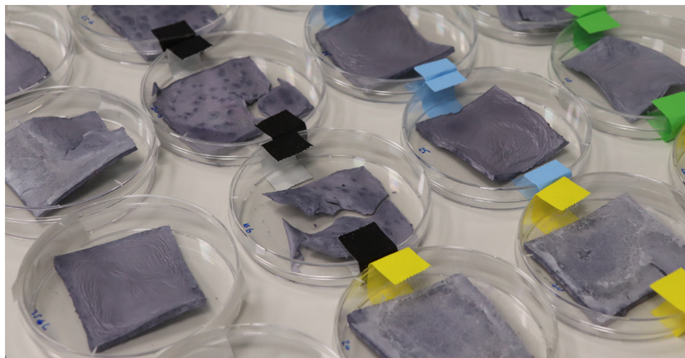


Figure 9.4 *- Various thicknesses of freeze-dried MAR medium*

Figure 9.5 *- Assembly of freeze-dried Flavobacteria and freeze-dried MAR medium*

9.1.2 Rehydration/ Activation

During the rehydration process, the guideline of using demi water equivalent to 40% of the original volume, as described in Chapter 8, was followed. It was observed that the thinnest samples did not exhibit any structural colour upon rehydration. Despite this, Flavobacteria growth was observed, resembling the colour of Flavobacteria growth in liquid medium (Figure 9.6). This suggests that the medium may have been over hydrated during the rehydration process.

In the experiments conducted on thicker variations of the medium, structural colour was indeed observed, but with variations in appearance and patterns, as depicted in Figures 9.7 to 9.10. Additionally, the growth rate of *C. lytica* also seemed to differ per sample. The comprehensive documentation of these experiments can be found in Appendix 9.2. Considering the objective of the living label to demonstrate relative change in response to temperature differences rather than achieving consistent aesthetics, the next experiment aimed to assess this aspect.

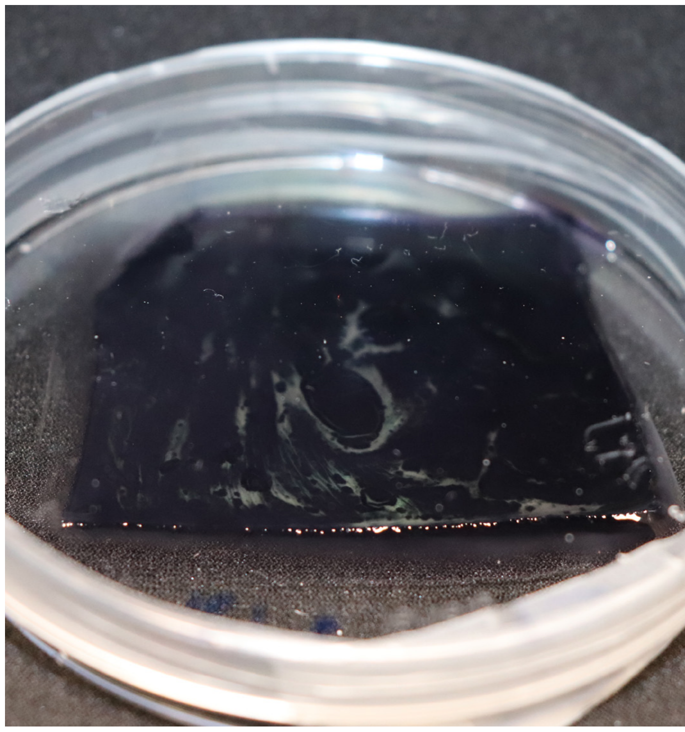


Figure 9.6 *- Rehydrated MAR medium and Flavobacteria (original volume MAR medium before freeze-drying 10 mL)*

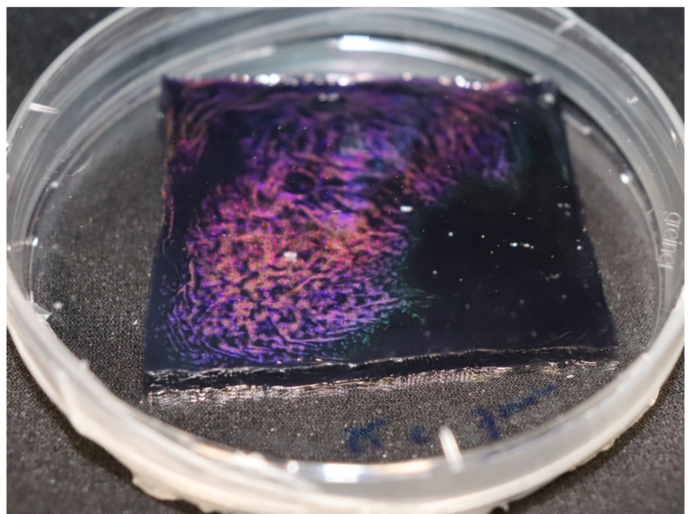


Figure 9.7*- Rehydrated MAR medium and Flavobacteria (original volume MAR medium before freeze-drying 25 mL)*

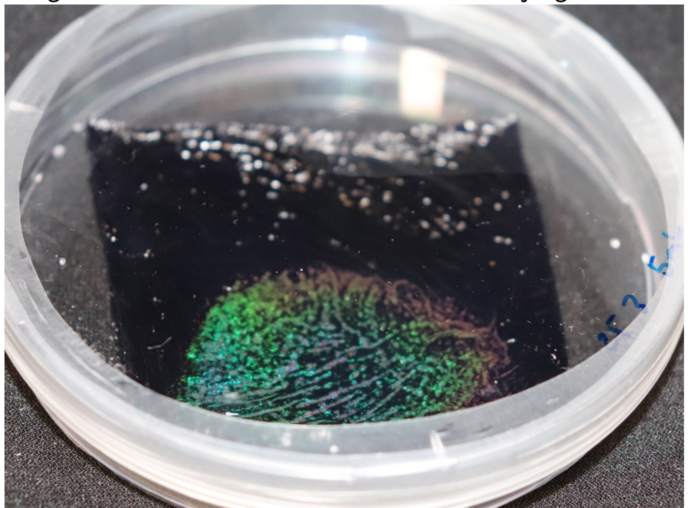


Figure 9.9 *- Rehydrated MAR medium and Flavobacteria (original volume MAR medium before freeze-drying 25 mL)*

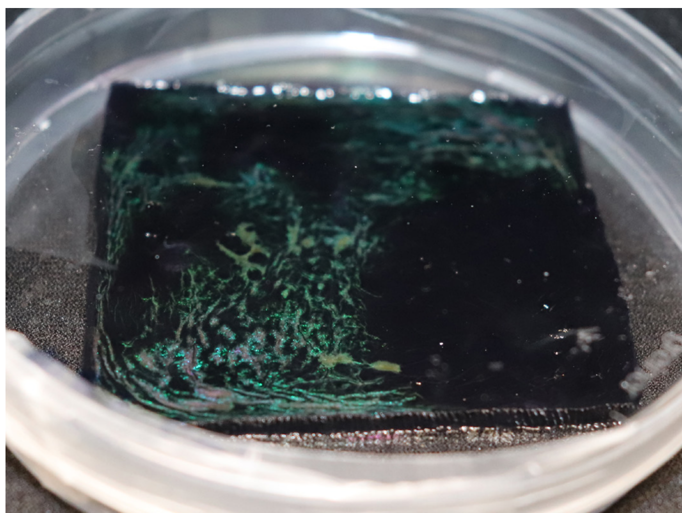


Figure 9.8 *- Rehydrated MAR medium and Flavobacteria (original volume MAR medium before freeze-drying 20 mL)*

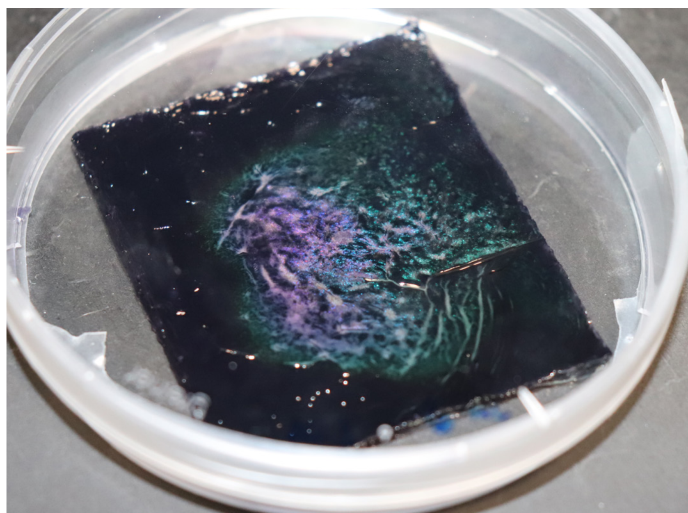


Figure 9.10 *- Rehydrated MAR medium and Flavobacteria (original volume MAR medium before freeze-drying 20 mL)*

9.1.3 Sensor Validation

The experiment of placing a rehydrated sample of freeze-dried Flavobacteria on freeze-dried MAR medium in the fridge to test its response to temperature variations showed unexpected results. In the specific sample chosen, there was no clear distinction in the living aesthetics between the colony after being kept in the fridge and the colony the days before at room temperature (Figures 9.11 to 9.14). This suggests that the combination of freeze-dried MAR medium with freeze-dried Flavobacteria may not be suitable for achieving the desired communicative value in the living label. Further investigation is necessary to understand why the behaviour of Flavobacteria in this design differs from their behaviour on regular MAR medium without freeze drying.

It is important to note that this outcome does not imply the inability of freeze-dried MAR medium and freeze-dried Flavobacteria to exhibit temperature-responsive properties altogether. However, in this particular combination and experimental setup, the expected distinction in living aesthetics was not observed. For this specific project however, returning to regular MAR medium and finding a different way to preserve it before the use phase, might be the most suitable direction to continue in.

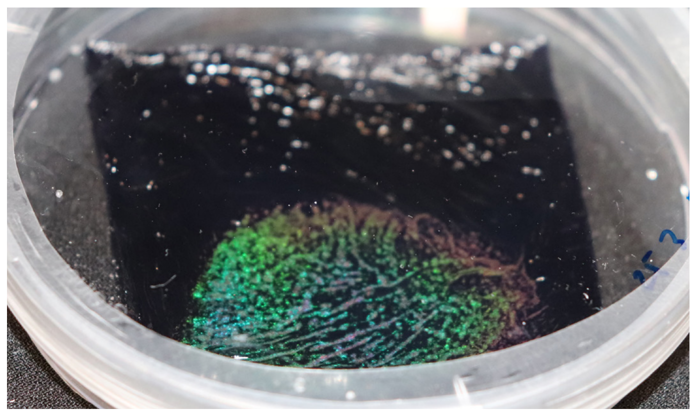


Figure 9.11 *- Rehydrated MAR medium and Flavobacteria, day 7, room temperature*

Fridge

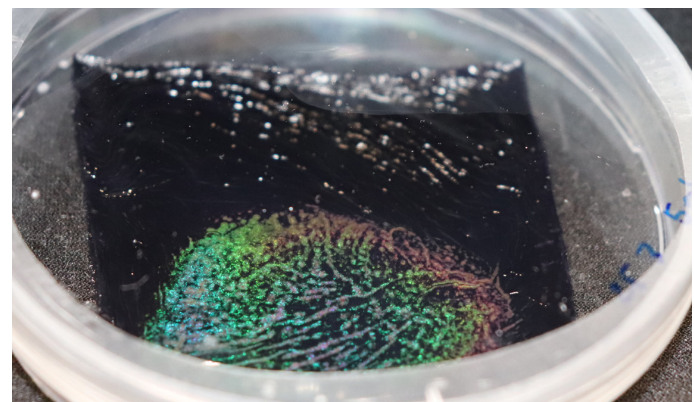


Figure 9.12 *- Rehydrated MAR medium and Flavobacteria, day 8, 4˚C*

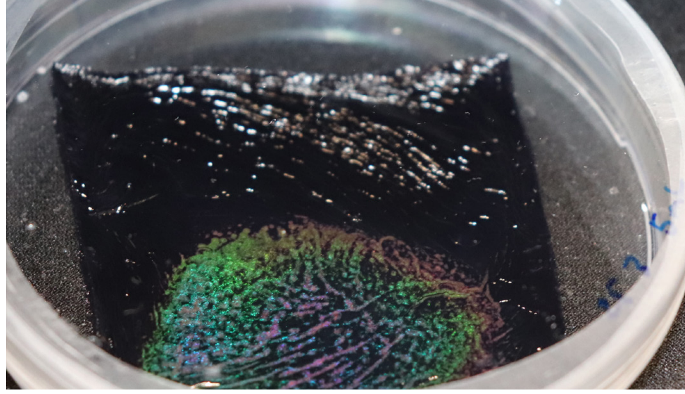


Figure 9.13 *- Rehydrated MAR medium and Flavobacteria, day 9, 4˚C*

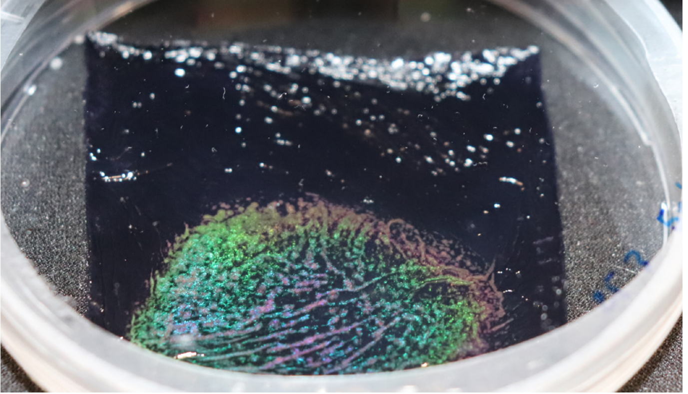


Figure 9.14 *- Rehydrated MAR medium and Flavobacteria, day 10, 4˚C*

9.2 Future Product Vision

The future vision of the living label incorporates living Flavobacteria into a flexible and attachable material, allowing it to serve as a temperature indicator (Figure 9.14). The label communicates changes in its surroundings through alterations in structural colour, providing visual cues to the user.

Before activation, the dry components are separated from the hydrated components within the label. MAR medium is sealed closed to ensure its hydration. With a layer that can be removed before activation, the MAR medium is separated from the freeze-dried Flavobacteria. This enables long-term storage without the need for specific environmental conditions. In the use phase, the separation layer is removed and the MAR medium hydrates the freeze-dried Flavobacteria, starting the growth.

The label is designed to be customizable for different use scenarios and specific temperature profiles required for the intended product or application.

An important aspect of the living label is its commitment to sustainability. The label is entirely biodegradable, ensuring minimal environmental impact after use. Additionally, users are provided with supplementary information to help interpret the living aesthetics of the Flavobacteria colony within the label, allowing them to understand the environmental signals being conveyed.

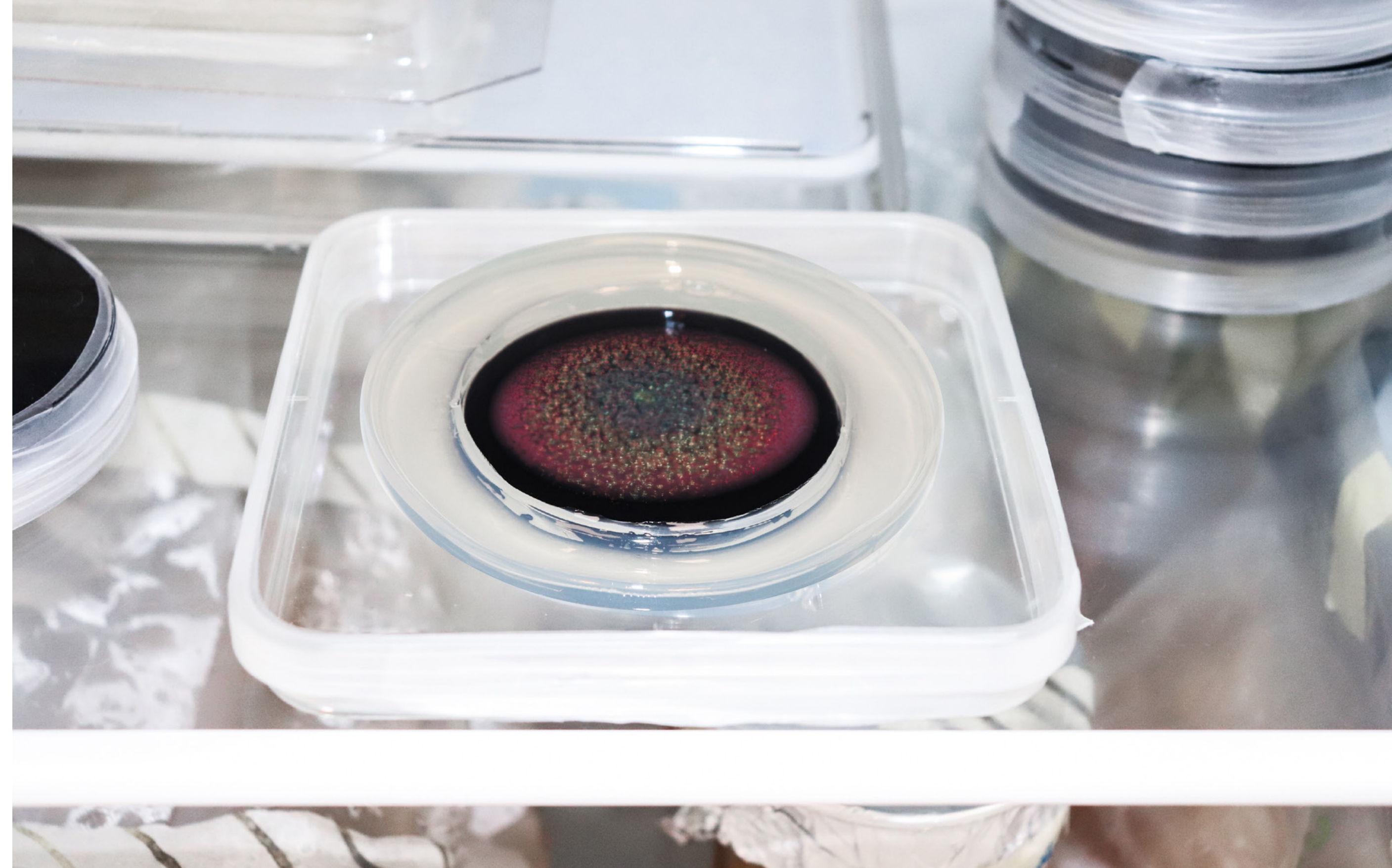


Figure 9.14 - *Future product vision of the living label*

9.3 Design Guidelines

Several protocols and methods were used to prepare the different parts of the living label. These, together with the insights and protocols established during the full scope of the project, can be found below in the form of guidelines for designers.

Design with Flavobacteria

The Guidelines

Species:
Cellulophaga lytica

Medium

MAR Medium (petri dishes)

To see structural colour & short term preservation

MAR Medium (Liquid)

To grow Flavobacteria & Long term preservation options

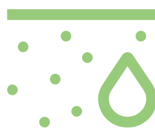
Stock

A stock of Flavobacteria can be kept by inoculating new petri dish with MAR medium once a week, freezing them in the -80°C freezer with glycerol or by freeze-drying the Flavobacteria.

Requirements

There are several requirements needed for Flavobacteria to grow and be able to make structural colour. These are listed below.

Oxygen
Flavobacteria are aerobic, so first and foremost they need oxygen.



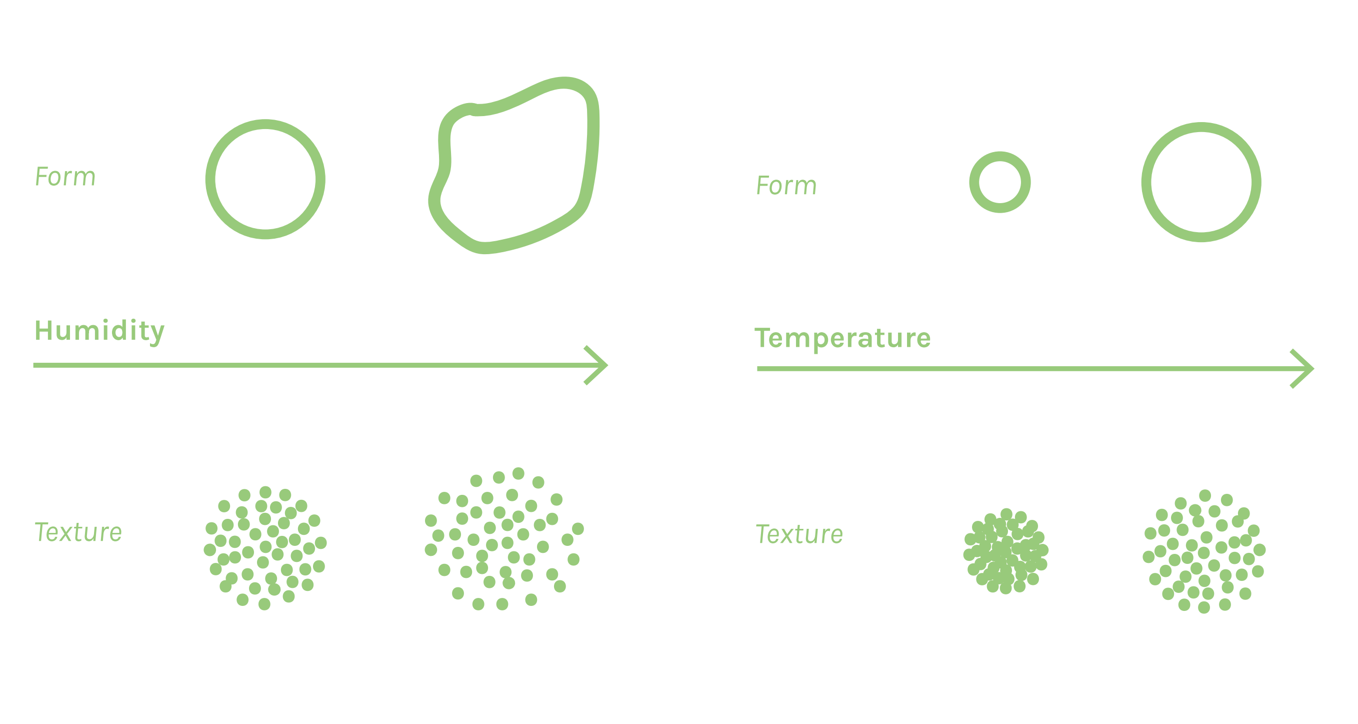
Salinity
The presence of essential seawater components is required for iridescence.

Humdity / Hydrogel
Humidity is needed for growth and a semi-solid, hydrated surface is needed to show structural colour. This surface is often created with agar as a gelling agent. The optimum agar concentration for iridescence is 1,5%.

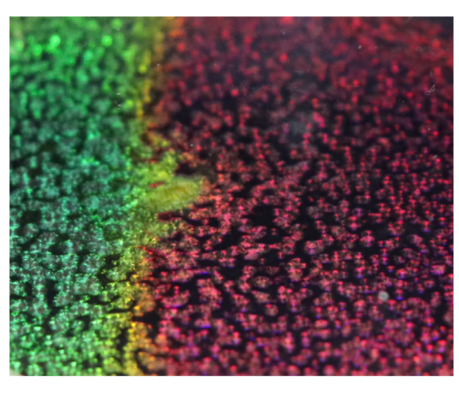
Nutrients
Nutrients are needed to help the colony grow.

Sources: (Kientz, Vukusic, et al., 2012)(Chapelais-Baron et al., 2018)(Kientz et al., 2012). (Groutars et al., 2022) (Kientz, Ducret, et al., 2012)(Kientz et al., 2013)

Living Aesthetics - Environmental Stimuli

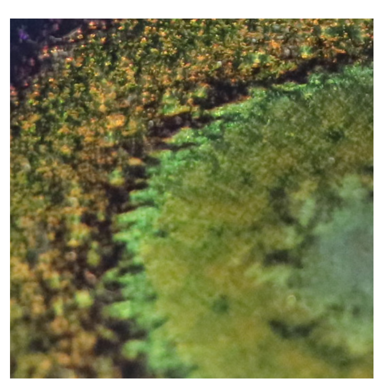


New Living Aesthetics (from this graduation project)



Glycerol

Glycerol was observed to affect the living aesthetics of a *C. lytica* colony by making them seem red.

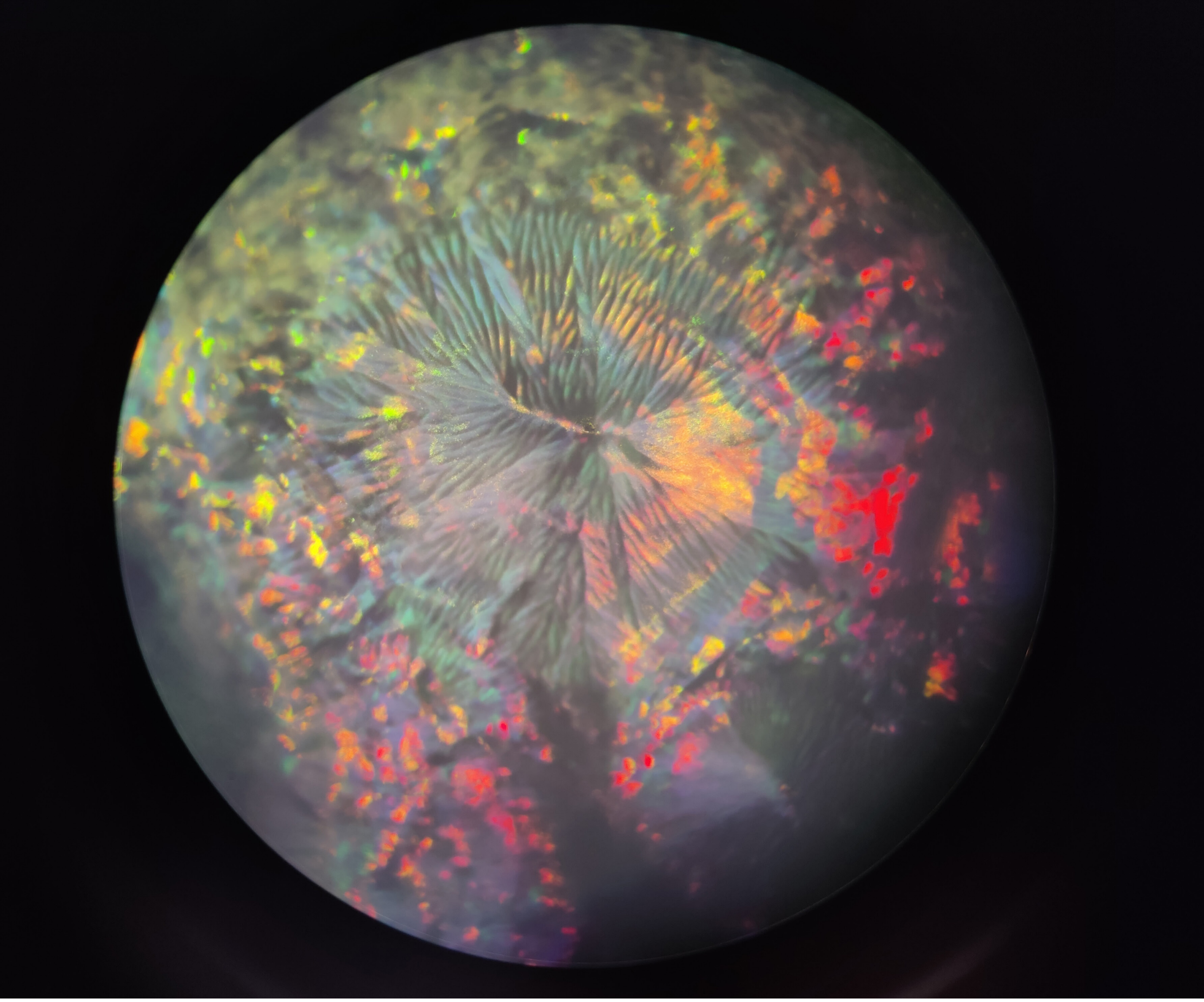


Temperature Rings

Temperature rings were observed when a *C. lytica* colony was alternated between temperature regimes.

New materials that *C. lytica* can grow under (from this graduation project)

All Bio4Life materials tested	*Hydrophobic coating*	*Biomaterials*
PLA NatureFlex PaperWise	PLA covered in beeswax	bio silicone (changed living aesthetic) agar foil (changed living aesthetic)



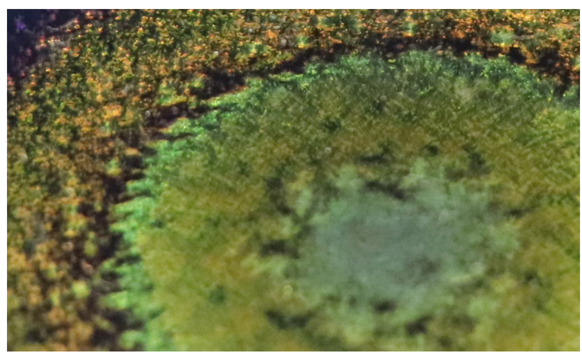
CH. 10

Recommendations

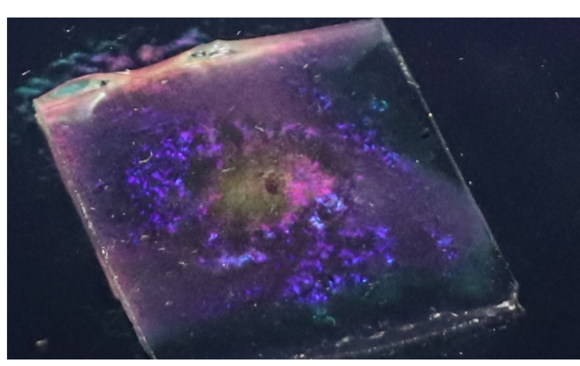
Introduction

Several parts of the living label coud use improvement in the future. The recommendations for future development of the living label as a sensor using Flavobacteria are outlined in this chapter.

Recommendations

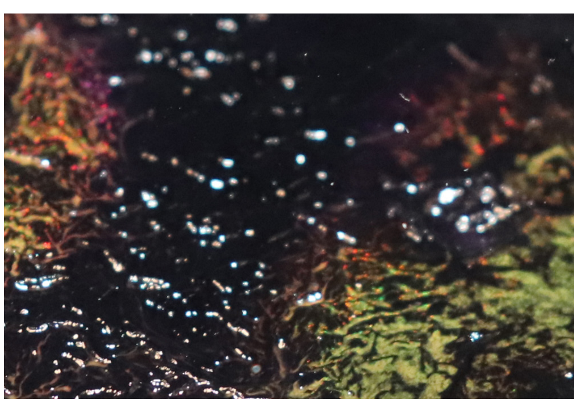


Activation Method
The protocol for freeze drying Flavobacteria should be improved to ensure accuracy in the quantity of bacteria freeze-dried. This accommodates a more precise way of experimenting with these freeze-dried bacteria and helps to understand the percentage of Flavobacteria that remain viable after the freeze drying process. Developing a method to determine the cell quantity before harvest will also help in controlling and standardising the amount of bacteria used for activation, leading to more consistent and reliable results.

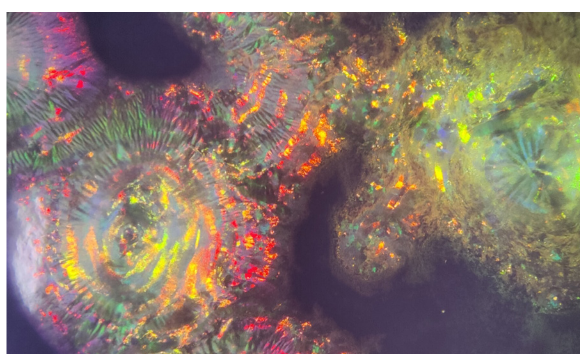


Top Layer
More research should be conducted into the properties of silicone and understand why Flavobacteria are able to generate structural colour under it. This can provide insights into which materials could be used as top layer in the future. Continuing the search for one material as top layer is advised. Firstly, since the label should be able to bend, adding physical headspace increases complexity since the top layer should not touch the Flavobacteria. Additionally, regarding the manufacturing process, a single material would simplify the assembly of the label.

Sensor
Further characterization of the temperature rings is necessary to fully understand how Flavobacteria can function as a sensor and how to interpret the living aesthetics of the colony in response to temperature changes. This will help in refining the sensor capabilities and improving the accuracy of temperature readings. Additionally, other stimuli like humidity could be looked into further for the living label as they are also known to influence the living aesthetics of a Flavobacteria colony.



Alternative Medium
The freeze-dried MAR medium should be further refined to achieve a smoother texture after rehydration. This will allow Flavobacteria to organise and grow in a desired colony pattern, enhancing the communicative value of the living label. Additional research is needed to explore techniques for achieving a smoother texture in the freeze-dried medium.



Agar Foil
The unexpected influence of glycerol in agar foil on the Flavobacteria colony suggests the need for further experimentation. Investigating the specific effects of glycerol on Flavobacteria growth and behaviour can provide valuable insights into its role for the living label and possibly expands our knowledge on Flavobacteria's optical structures and

By addressing these recommendations, the potential of Flavobacteria as a sensor can be further explored, and improvements can be made to enhance the performance, reliability, and aesthetic qualities of the living label.

Acknowledgements

First and foremost, I would like to thank my supervisory team, Joana Martins and Clarice Risseeuw. Your time, effort and patience have helped me to get the most out of this project and has made this a very rewarding experience. Next to that, I would like to thank the both of you for the shared enthusiasm for this project and for always providing me with an honest review of my work.

Additionally, I would like to thank Joren Wierenga for the patience and time and answering all of my questions related to the BioLab (and there were many). This was a major contributor to being able to do the experiments that I did, and made for a very enjoyable experience in the lab over the last weeks.

I would also like to thank the experts who were involved in the project, Bio4Life and Hoekmine, for the company visits and the availability to ask questions.

Lastly, this research project entitled Living Circular Labels [KIEM.CIE.06.007] was funded by the KIEM programme of "Regieorgaan SIA".

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Appendix

Living Labels

Evy Murraij
July 21st, 2023

Delft University of Technology,
Faculty Industrial Design Engineering
Landbergstraat 15, 2628 CE Delft

Living Labels
Appendix

21-07-2023
Evy Murraij
[5496357]

Cover image: C. lytica under microscope

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Personal Project Brief - IDE Master Graduation

Living Labels — project title

Please state the title of your graduation project (above) and the start date and end date (below). Keep the title compact and simple. Do not use abbreviations. The remainder of this document allows you to define and clarify your graduation project.

start date 13 - 02 - 2023 — 21 - 07 - 2023 end date

INTRODUCTION **

Please describe, the context of your project, and address the main stakeholders (interests) within this context in a concise yet complete manner. Who are involved, what do they value and how do they currently operate within the given context? What are the main opportunities and limitations you are currently aware of (cultural- and social norms, resources (time, money,...), technology, ...).

Within the field of biodesign, designers, artists and scientists are collaborating with living organisms to produce new materials with ecological benefits. In a society where sustainability becomes a more important topic every day, interest is growing towards design solutions that take the planet into account. Not only is design with living artifacts an opportunity to be more sustainable, when keeping organisms alive in the final design such artifacts also offer novel responsive behaviour and interaction possibilities. Biodesign enables new ways of living in collaboration with nature. Several organisms are being used for biodesign because of their characteristics. An example are organisms like bio luminescent algae with their light emitting possibilities[1] or algae for air, soil or water "cleaning"[2].
The focus of this graduation project is the living organism Flavobacteria. Flavobacteria are marine organisms that produce vivid, angle-dependent colour as their cells organize into structures that interact with light. Their colour changes over time and in relation to environmental factors, such as temperature and humidity[3]. This specific characteristic of Flavobacteria is a very interesting aspect that can open up opportunities for integration with every day objects like, for instance, a living sensor that can have the potential to replace the existing electronic sensors.

Until now, the cell-organization of these Flavobacteria, resulting in the structural colour, has only been observed in the laboratory environment which makes it a challenging material to use in the design of everyday objects. But what if Flavobacteria's capability to form structural colour could be used outside the lab to fit a design purpose? We aim to embed Flavobacteria in labels that can be used as stand-alone sensors (e.g., to communicate temperature and humidity change, or existence of other organisms within an environment) or that can be attached to products which are subjected and sensitive to environmental changes and passing of time (e.g., goods with short shelf life, plants, etc.). The label used for this will be a biodegradable label that comes from a company called Bio4Life[4]. Next to that the microbiologists from Hoekmine[5] can also be consulted over the course of this graduation project. Hoekmine is a company that works with Flavobacteria. In collaboration with designers, they have been researching the potential of flavobacteria for producing sustainable colorants to be applied on everyday products. They also envision biosensors, which would minimize the use of increasingly demanded electronic sensors, and thus, the implementation of scarce and toxic materials that are not most of the time not properly collected at end-of-life[6].

[1]Barati, B., Karana, E., Pont, S., & van Dortmont, T. (2021). LIVING LIGHT INTERFACES —AN EXPLORATION OF BIOLUMINESCENCE AESTHETICS. Designing Interactive Systems Conference 2021. https://doi.org/10.1145/3461778.3462038
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Personal Project Brief - IDE Master Graduation

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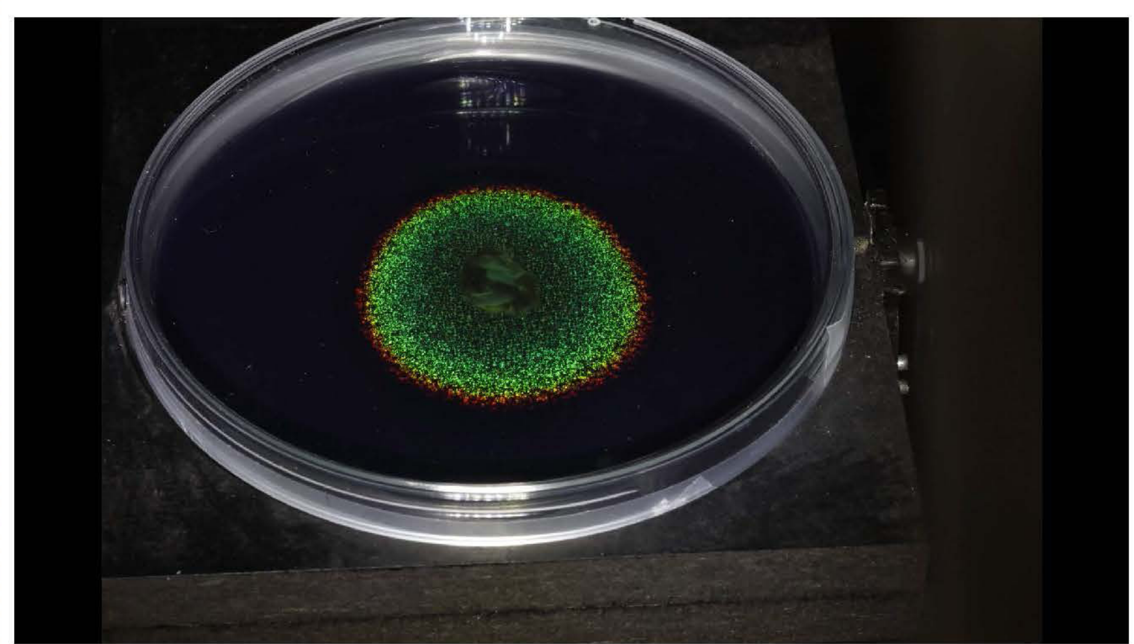


image / figure 1: Flavobacteria growing in a Petri-dish.

image / figure 2: Flexible Habitat for Flavobacteria.

Personal Project Brief - IDE Master Graduation

PROBLEM DEFINITION **
Limit and define the scope and solution space of your project to one that is manageable within one Master Graduation Project of 30 EC (= 20 full time weeks or 100 working days) and clearly indicate what issue(s) should be addressed in this project.

One of the major challenges when working with living materials is to maintain and grow, in sufficient yield, living organisms outside the optimal laboratory conditions. In addition, the desired biological trait that is being explored to fit the design purpose (e.g., structural colour) also needs to be kept. Understanding how to maintain Flavobacteria cells, organised to form structural colour, outside the laboratory can be the steppingstone to make the entry of these microorganisms into the world of design.

To achieve this, we aim to integrate living Flavobacteria into a flexible, sustainable materiality, creating labels that communicate environmental signals through changes in their structural colour. Secondly, we also intend to explore 'activation' methods of Flavobacteria growth, to avoid its growth in the label until it is activated by the users.
By doing this we aim to bridge the gap between microbiology and embodiment design and contribute to the development of a circular economy where technology and organic systems merge in the design of living circular labels.

ASSIGNMENT **
State in 2 or 3 sentences what you are going to research, design, create and / or generate, that will solve (part of) the issue(s) pointed out in "problem definition". Then illustrate this assignment by indicating what kind of solution you expect and / or aim to deliver, for instance: a product, a product-service combination, a strategy illustrated through product or product-service combination ideas, ... In case of a Specialisation and/or Annotation, make sure the assignment reflects this/these.

During this project, we will encapsulate Flavobacteria in flexible, sustainable materiality, creating labels that communicate environmental signals through vivid colour. Secondly, we aim to identify possibilities for activating the growth of Flavobacteria in this specific medium.

The expected solution of this graduation project is a biodegradable label, with materials from Bio4Life, in which the Flavobacteria can survive and be used as a living colour interface outside of a laboratory environment. Secondly, a way to activate Flavobacteria's growth. Therefore the outcome will be a proof-of-concept and design guidelines for biodesigners to create living labels with Flavobacteria.

Initials & Name E.A.J. Murraij Student number 5496357

Title of Project Living Labels

Personal Project Brief - IDE Master Graduation

PLANNING AND APPROACH **

Include a Gantt Chart (replace the example below - more examples can be found in Manual 2) that shows the different phases of your project, deliverables you have in mind, meetings, and how you plan to spend your time. Please note that all activities should fit within the given net time of 30 EC = 20 full time weeks or 100 working days, and your planning should include a kick-off meeting, mid-term meeting, green light meeting and graduation ceremony. Illustrate your Gantt Chart by, for instance, explaining your approach, and please indicate periods of part-time activities and/or periods of not spending time on your graduation project, if any, for instance because of holidays or parallel activities.

start date 13 - 2 - 2023

21 - 7 - 2023 end date

Start date: 13/02/2023	36 hours per week graduation, 22 weeks																						
End date: 21/07/2023																							
TU Delft Week	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10			
Calendar week	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Project Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Internal and External Kickoff																							
Literature Research																							
Experiments																							
Contact with Bio4Life																							
Contact with Hoekmine										Break													
Mid Term Evaluation																							
Work on report																							
Green Light																							
Graduation																							

Personal Project Brief - IDE Master Graduation

MOTIVATION AND PERSONAL AMBITIONS

Explain why you set up this project, what competences you want to prove and learn. For example: acquired competences from your MSc programme, the elective semester, extra-curricular activities (etc.) and point out the competences you have yet developed. Optionally, describe which personal learning ambitions you explicitly want to address in this project, on top of the learning objectives of the Graduation Project, such as: in depth knowledge a on specific subject, broadening your competences or experimenting with a specific tool and/or methodology, ... Stick to no more than five ambitions.

Over the course of my studies I have experienced that my interests are very broad. However, the common factor in all of these interests is sustainability. Through my bachelor and master I have had the opportunity to experience ways in which sustainability can be incorporated into design. I have learned about sustainable design strategies, concepts like cradle to cradle, more sustainable materials and also learned how to prevent (unintentional) green washing. However, I could never really get excited about all of these directions. For me, less bad was still not good enough.

Due to this, my interest was immediately sparked by the course "Fundamentals of Biodesign" that was new in the elective space of the IDE master. This fascination continued when I started to learn more about biodesign in the course. The idea of co-existing with nature is extremely inspirational for me and finally talks about a 'good' direction instead of one that is less bad. It made me think, why do we actually kill every material that we use? I believe that biodesign is the sustainability direction of the future and this is why I want to be a part of the process towards that future.

This graduation project is the perfect opportunity for me to acquire the skills that are needed to work in the field of biodesign. Since it differs from traditional design in the fact that the materials used are actually alive, this asks for a different approach. I have gained some understanding of working in the lab and basic biodesign principles in the courses Fundamentals of Biodesign and Design with Living artifacts. Next to that, I have also gained experience in a company focused on biodesign, namely 'Loop'. This company was started by an IDE alumni called Bob Hendrikx. I worked there several times as a 'grower'. This graduation project will be the next step because it will help me understand how I could work in biodesign by myself and teach me the necessary skills and approach.

Personally, another goal I have is to gain a much better understanding of the biology of our world. More in depth knowledge of this will help me in my biodesign journey and is just interesting in general. Lastly as mentioned before, I would like to become confident with working in a laboratory environment.

FINAL COMMENTS

In case your project brief needs final comments, please add any information you think is relevant.

Initials & Name E.A.J. Murraij Student number 5496357

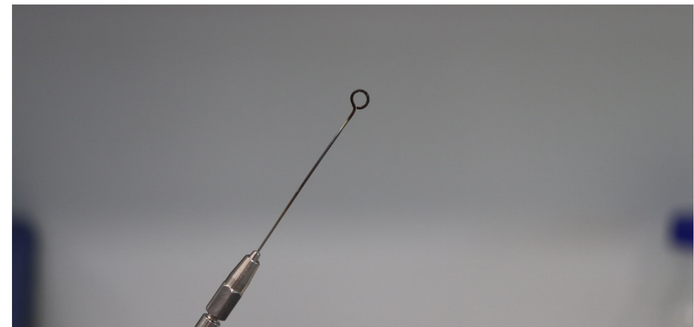
Title of Project Living Labels

Lab Coat

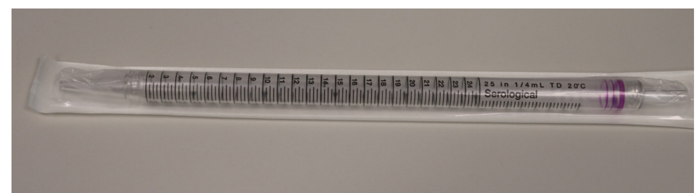
Parafilm

Petri Dish

Precise pipette



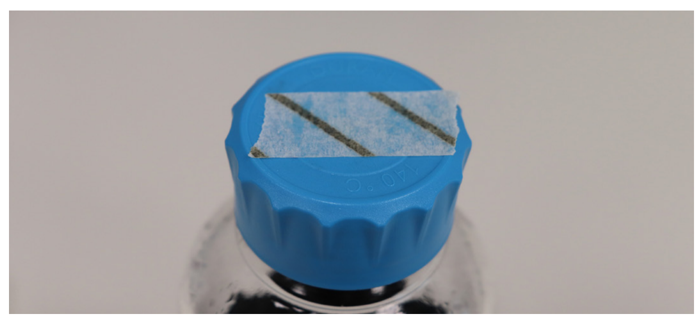
Inoculation Loop



Sterile Pipette

Pipette controller

500 mL Flask

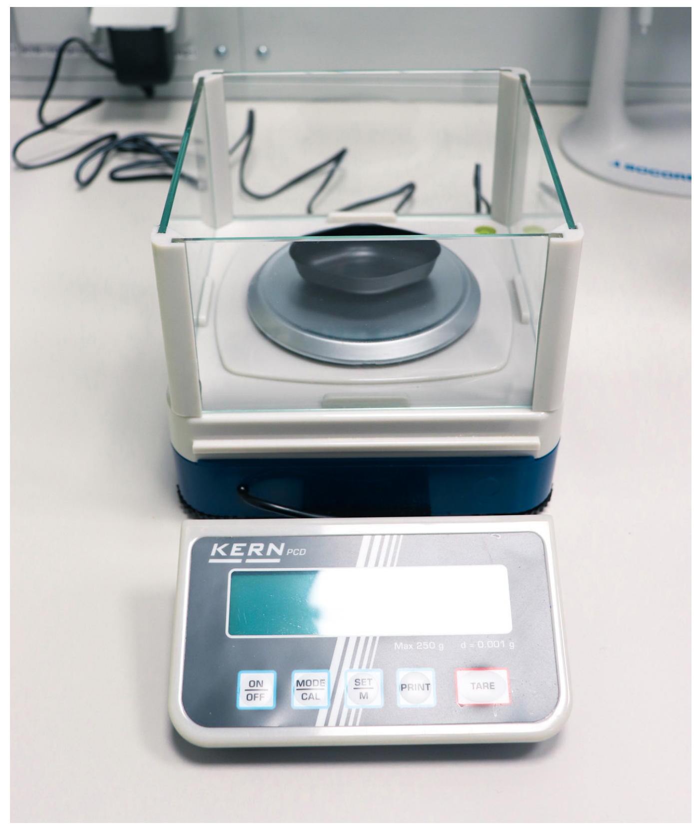


Autoclave Tape

Spoons and Spatula’s

Autoclave (for sterilization)

Sterile Tubes



Scale

A.2 / BioLab Equipment

Laminar Airflow Cabinet

Vortex

Freeze Dryer

Centrifuge

A.3 / MAR Medium
Recipe and Preparation

MAR Medium ingredients (400mL):

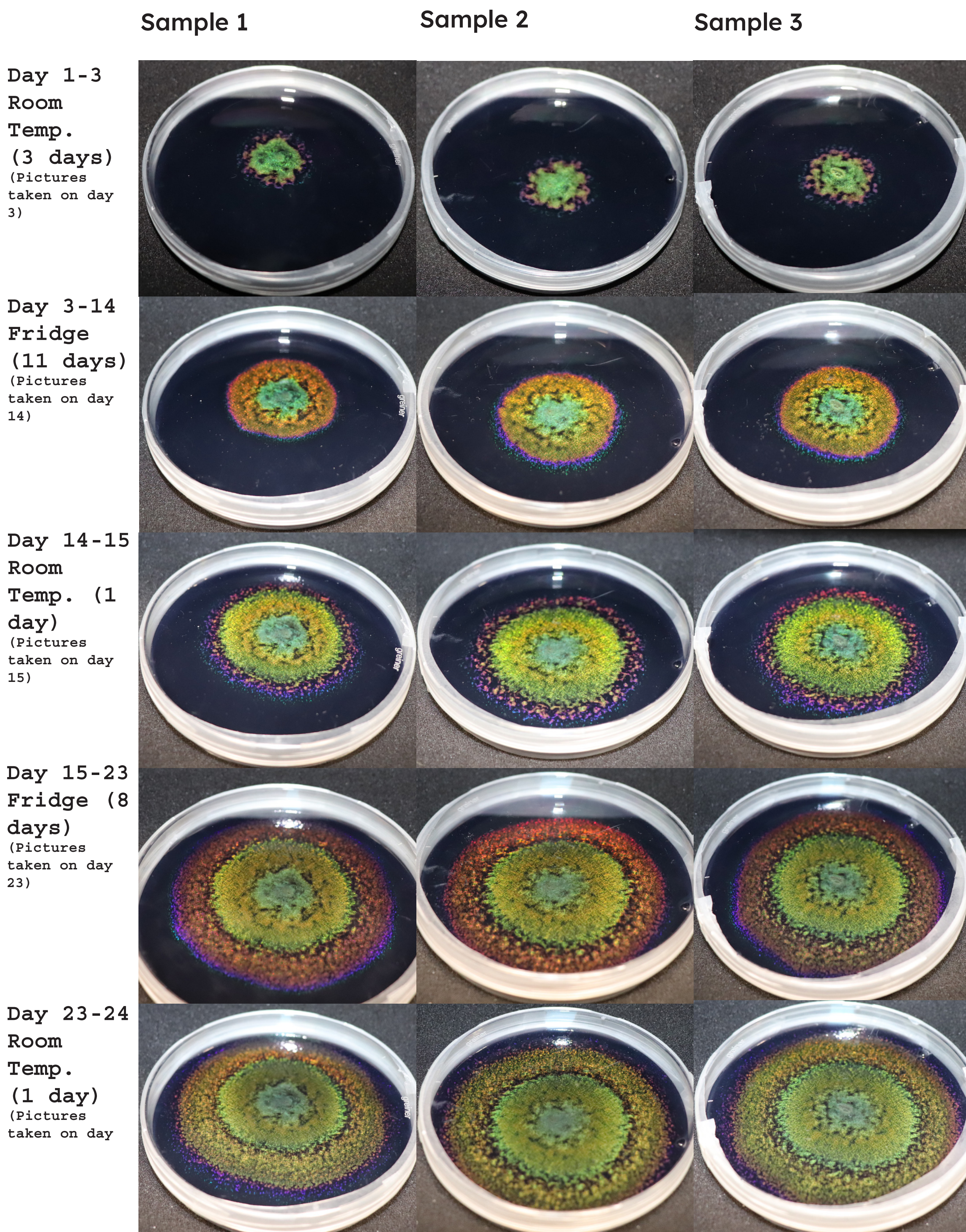
Agar	6 gr
MgS04	0,125 g/L (400 microliter pipette)
KNO3	0,025 g/L (400 microliter pipette)
Nigrosine	10mL
Peptone	1 gr
Yeast	0,1 gr
sea salt	13 gr
demi water	390 gr

Tools
10 mL pipette
Pipette controller
3 spoons
500 mL bottle
Scale

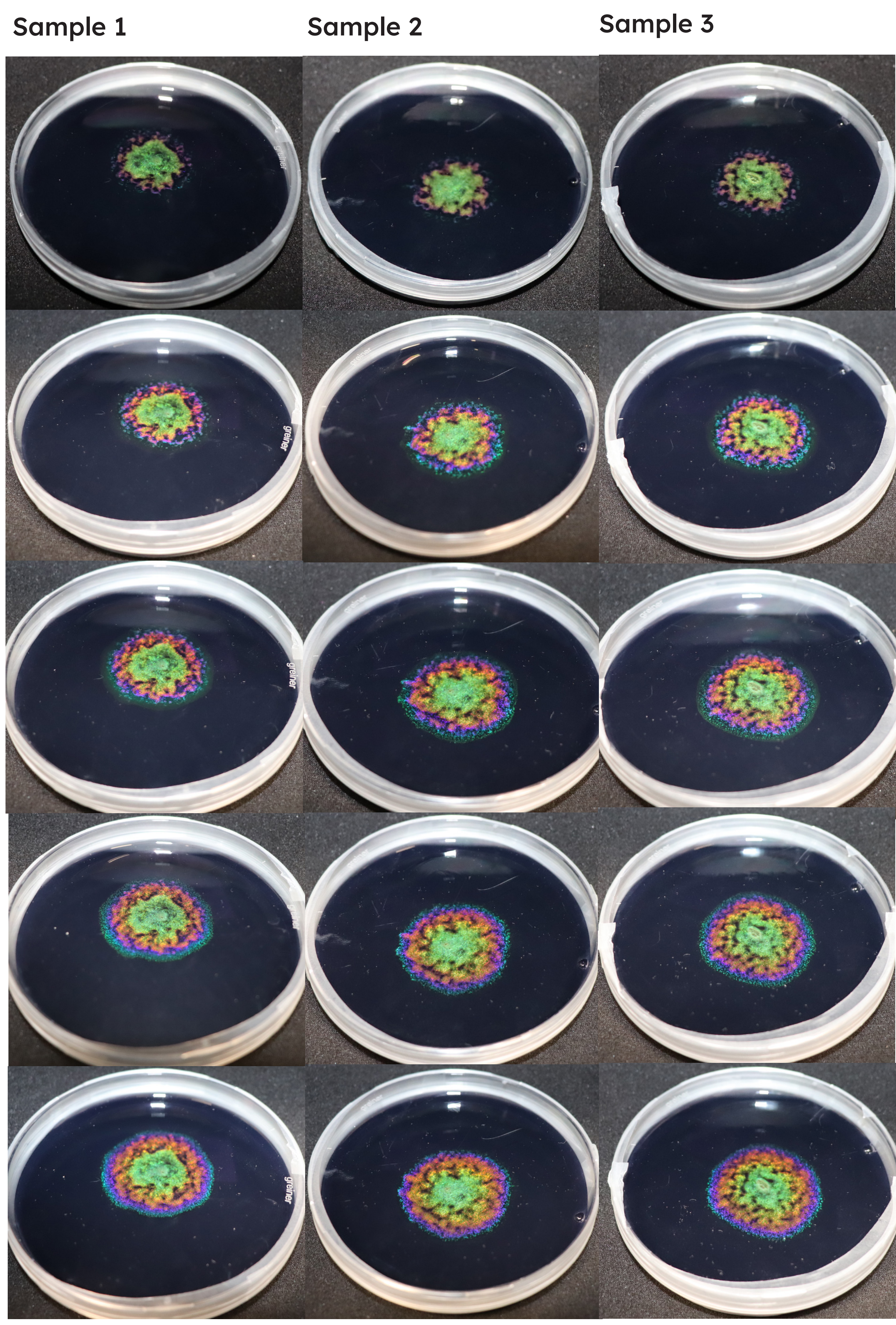
Method
1. Clean workspace
2. Take out all of the ingredients
3. Put name and autoclave tape on bottle of 500mL
4. Weigh yeast in weighing boat and add to bottle
5. Weigh peptone in weighing boat and add to bottle
6. Pipette MgS04 into bottle
7. Pipette KNO3 into bottle
8. Pipette 10 mL nigrosine into bottle
9. Put bottle on scale and tare
10. add 13 gr of sea salt
11. add 6 gr of agar
12. add 390 gr of demi water (fill to 400mL)
13. Close bottle

Autoclaving
1. put cap loose on bottle
2. Put in autoclave
3. Set temperature to 121 degrees celsius
4. Set time to 30 min
5. While on temperatuur option press ‘start’

A.4 / Fridge Test
Results Summary



A.4 / Fridge Test
Full Documentation



Day 3
Room Temp.
(March 31st)

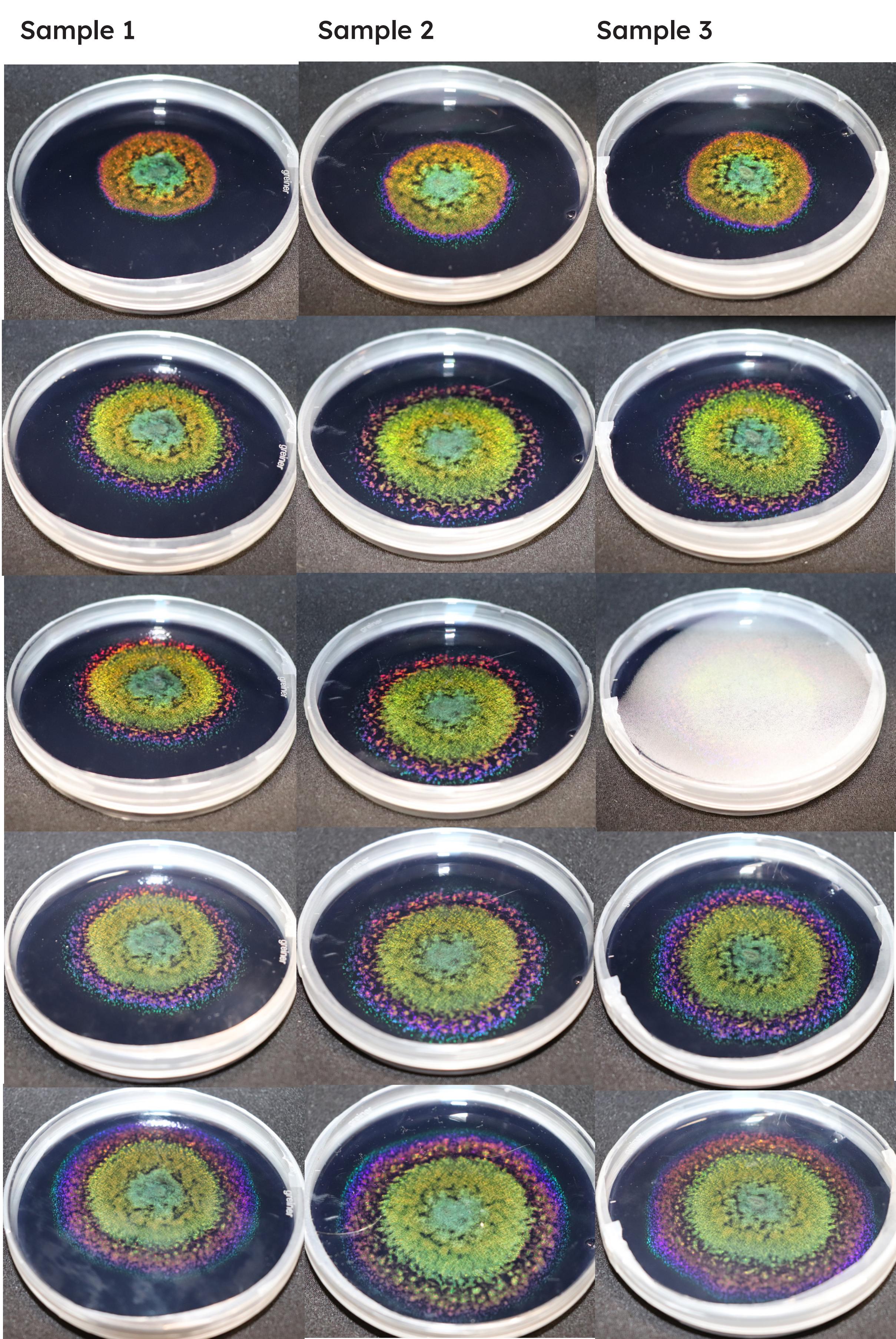
Day 6
Fridge
(April 3rd)

Day 7
Fridge
(April 4th)

Day 8
Fridge
(April 5th)

Day 9
Fridge
(April 6th)

A.4 / Fridge Test
Full Documentation



Day 14
Fridge
(April 11th)

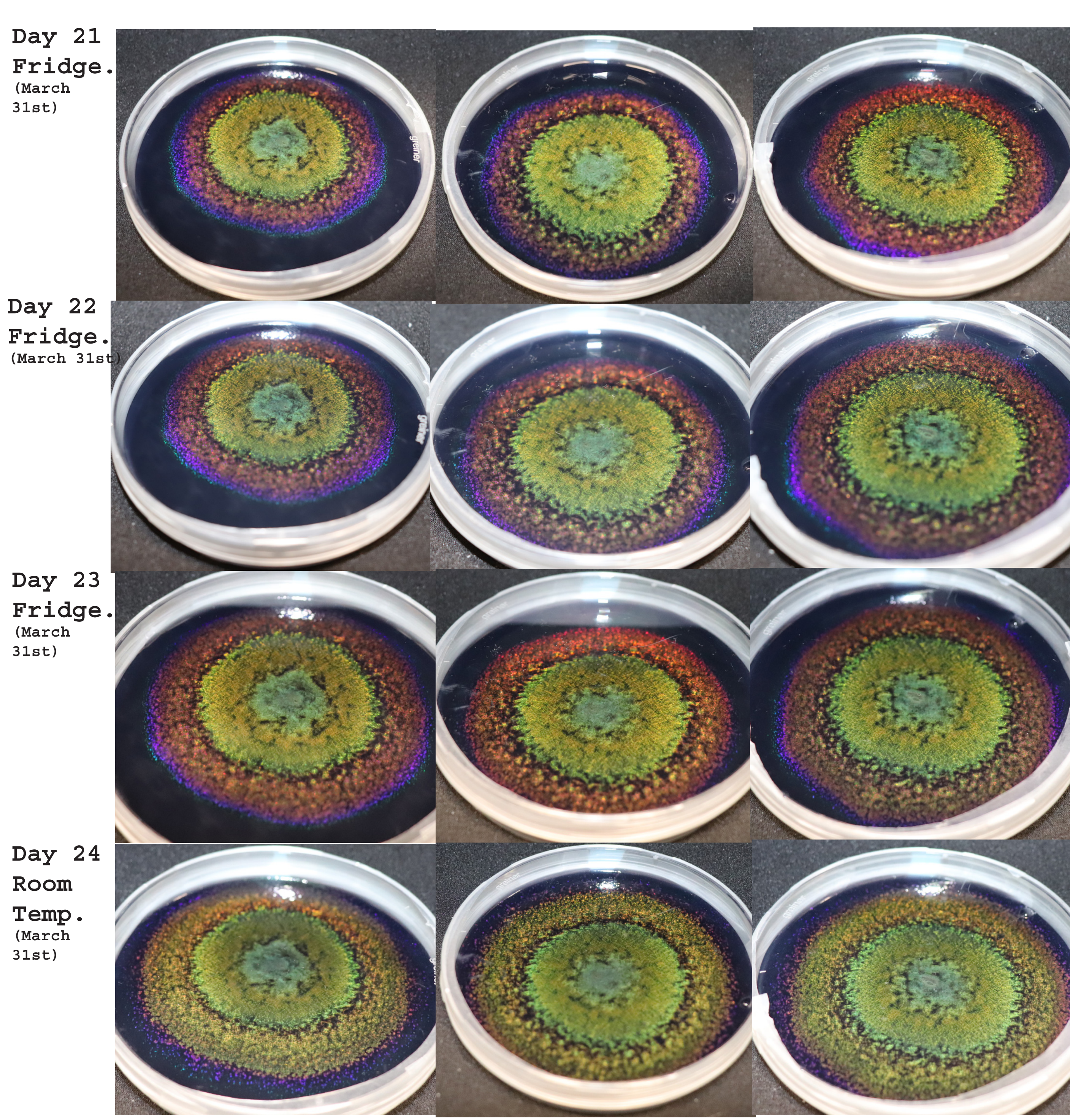
Day 15
Room Temp.
(April 12th)

Day 16
Fridge.
(April 13th)

Day 17
Fridge.
(April 14th)

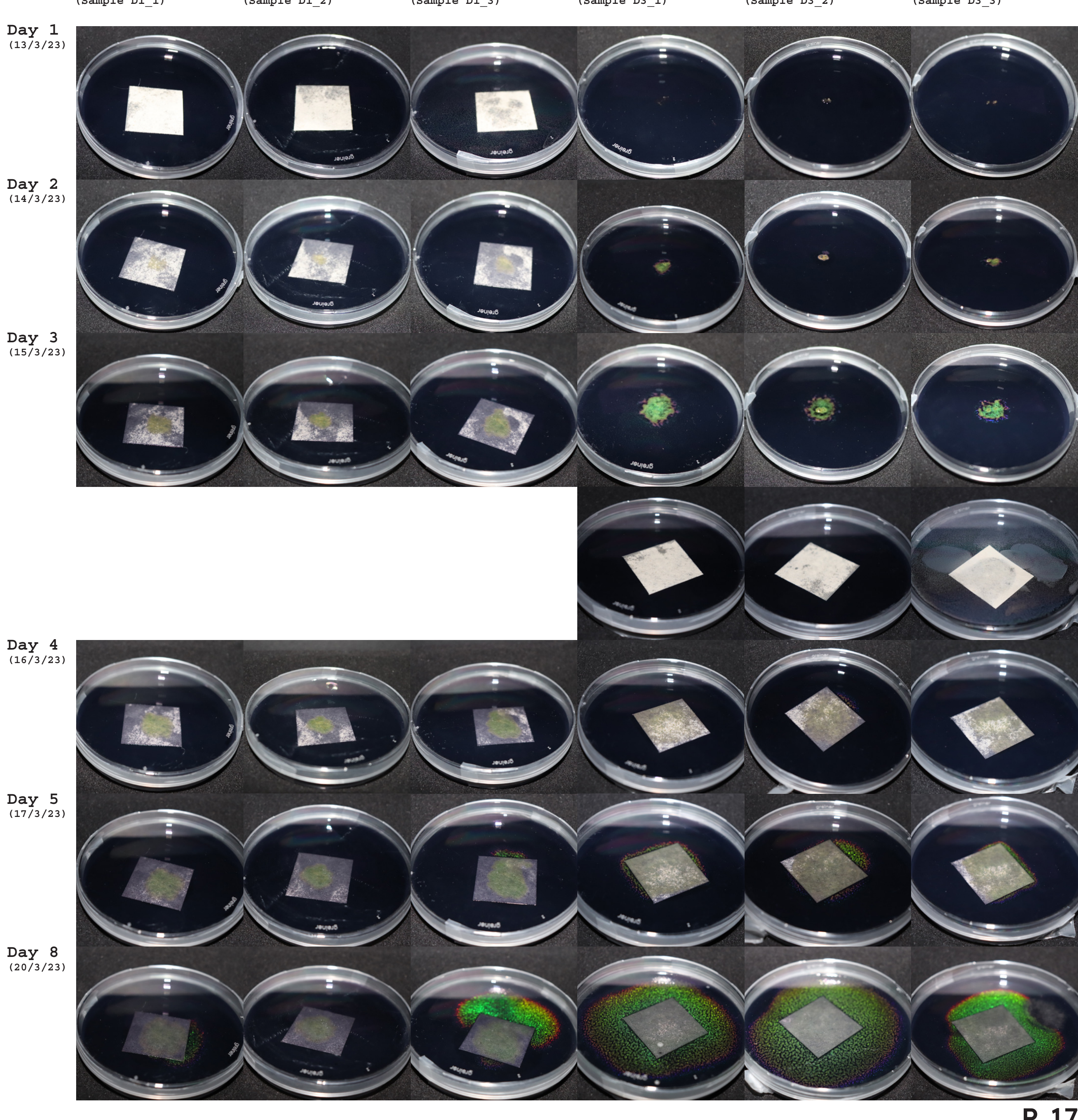
Day 20
Fridge.
(March 31st)

A.4 / Fridge Test
Full Documentation

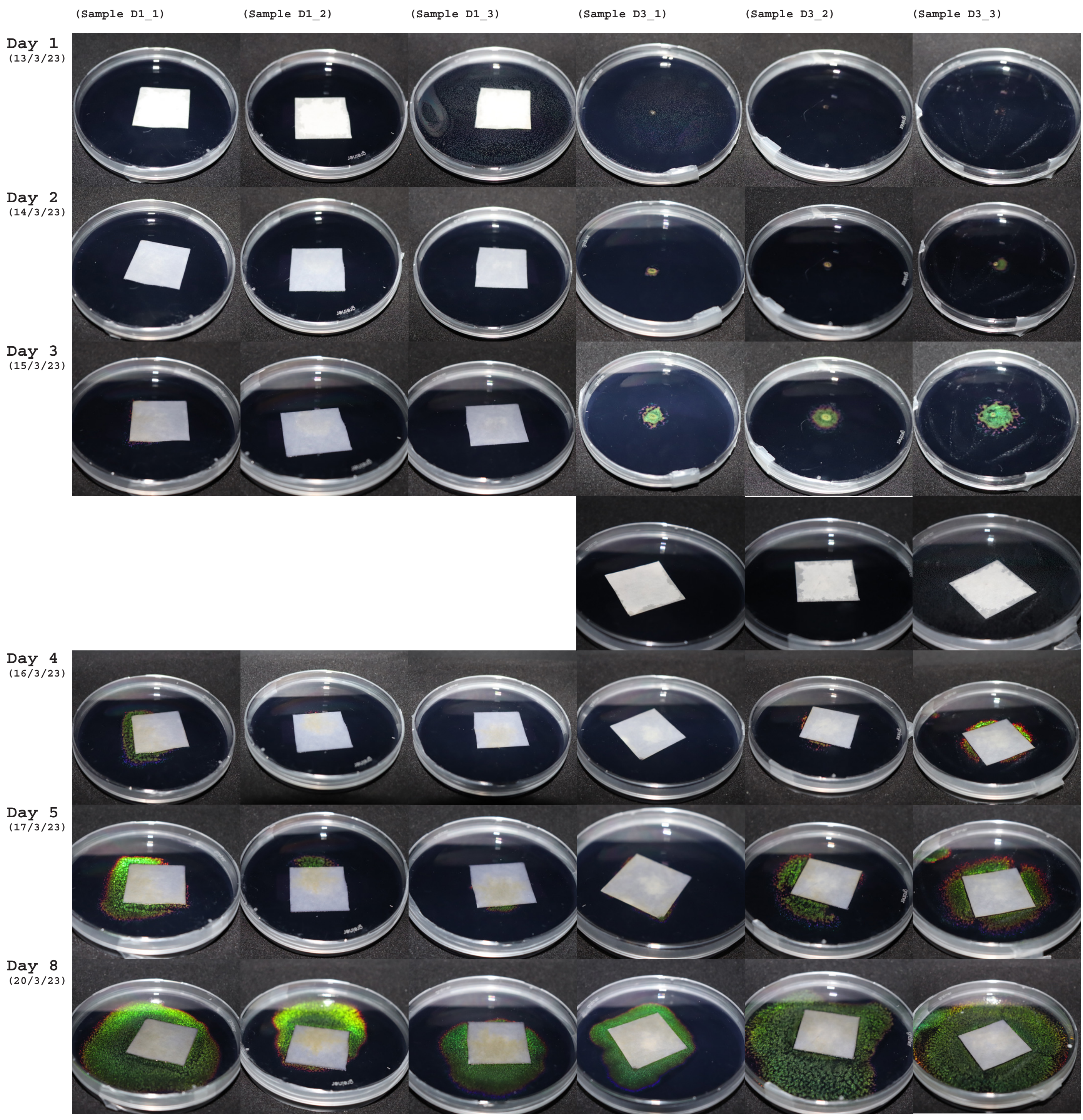


A.5.1 / Paperwise

Bio4Life Material

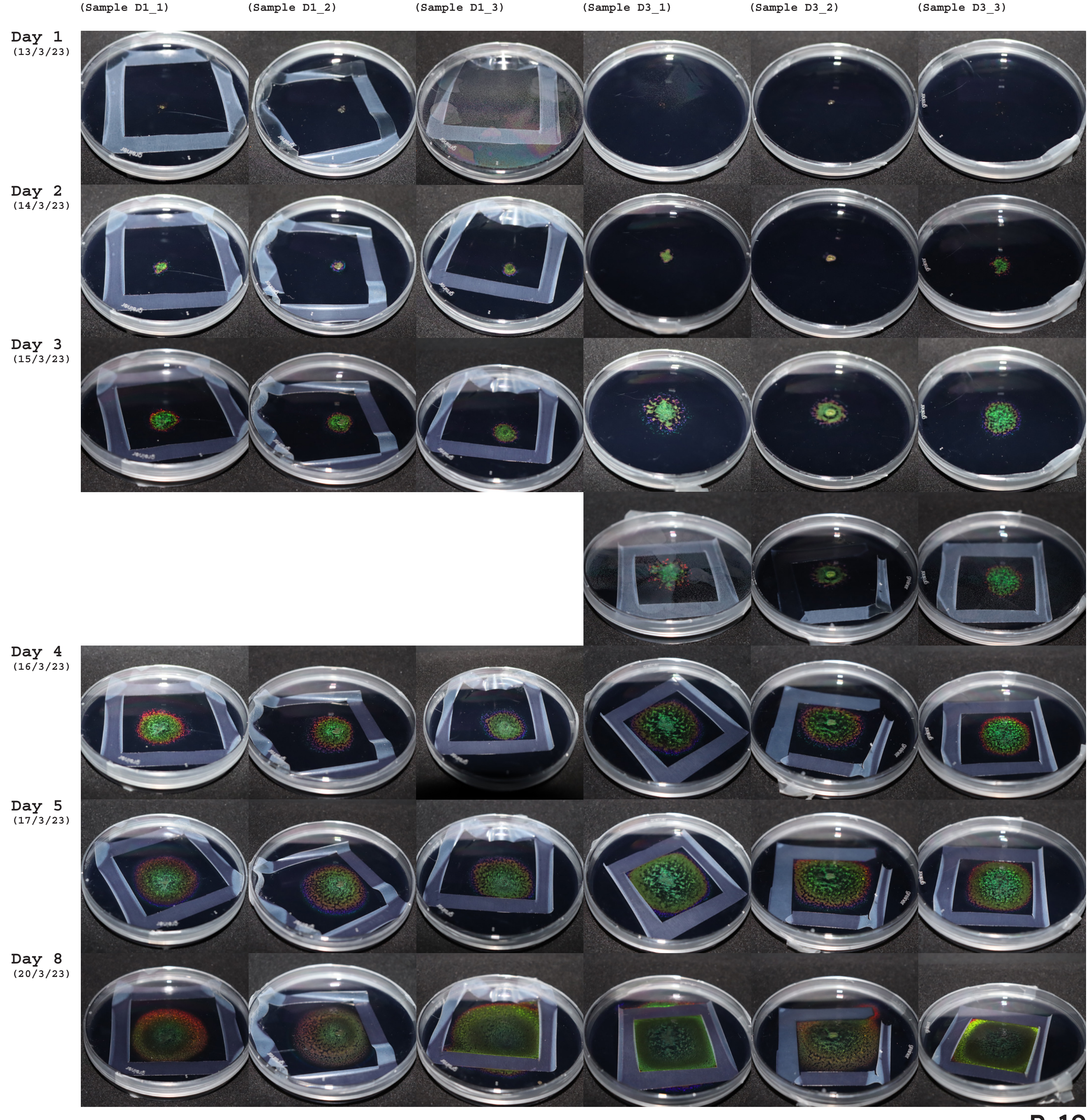


A.5.2 / HP Ink
Bio4Life Material



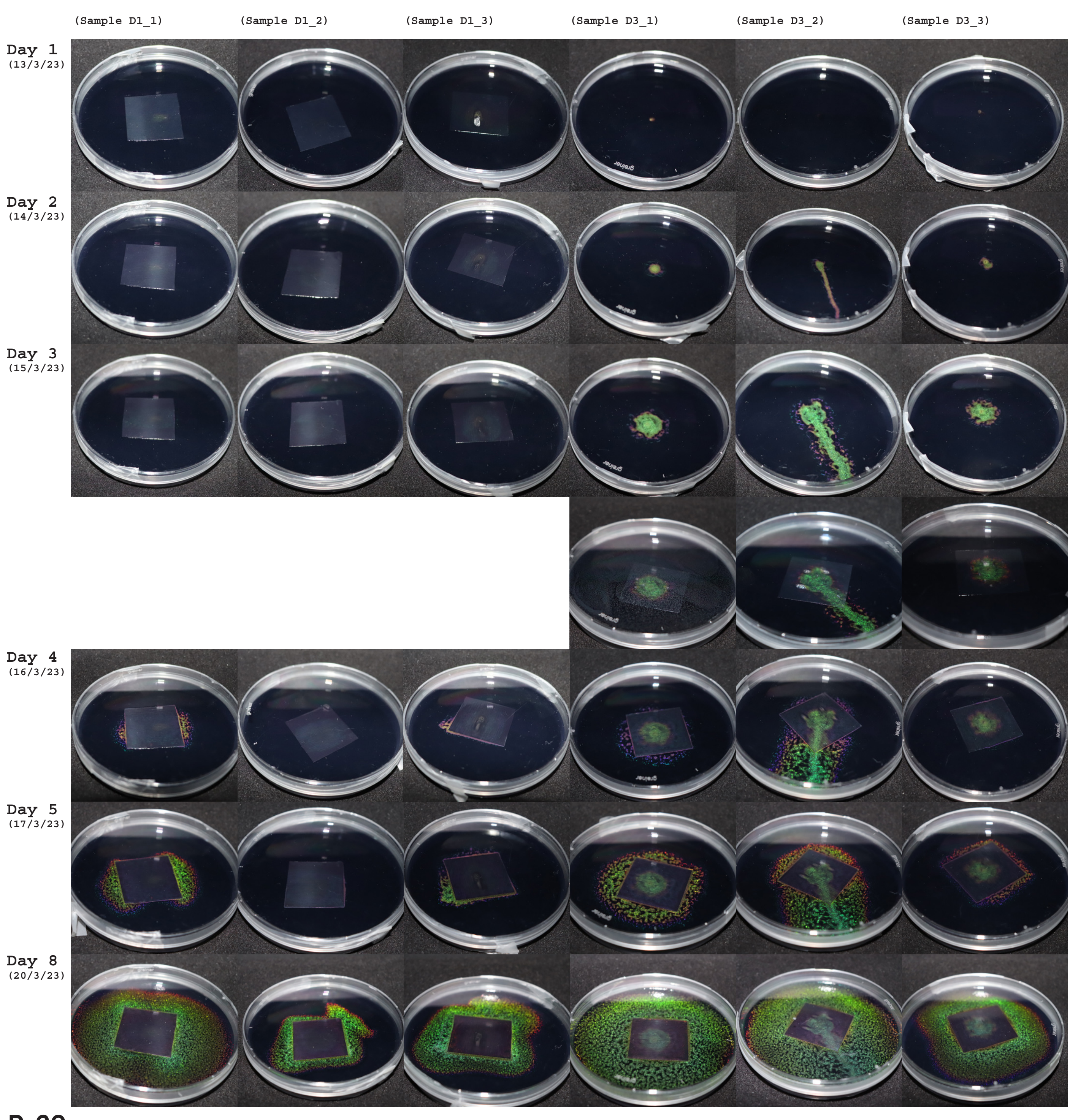
A.5.3 / BioTak

Bio4Life Material

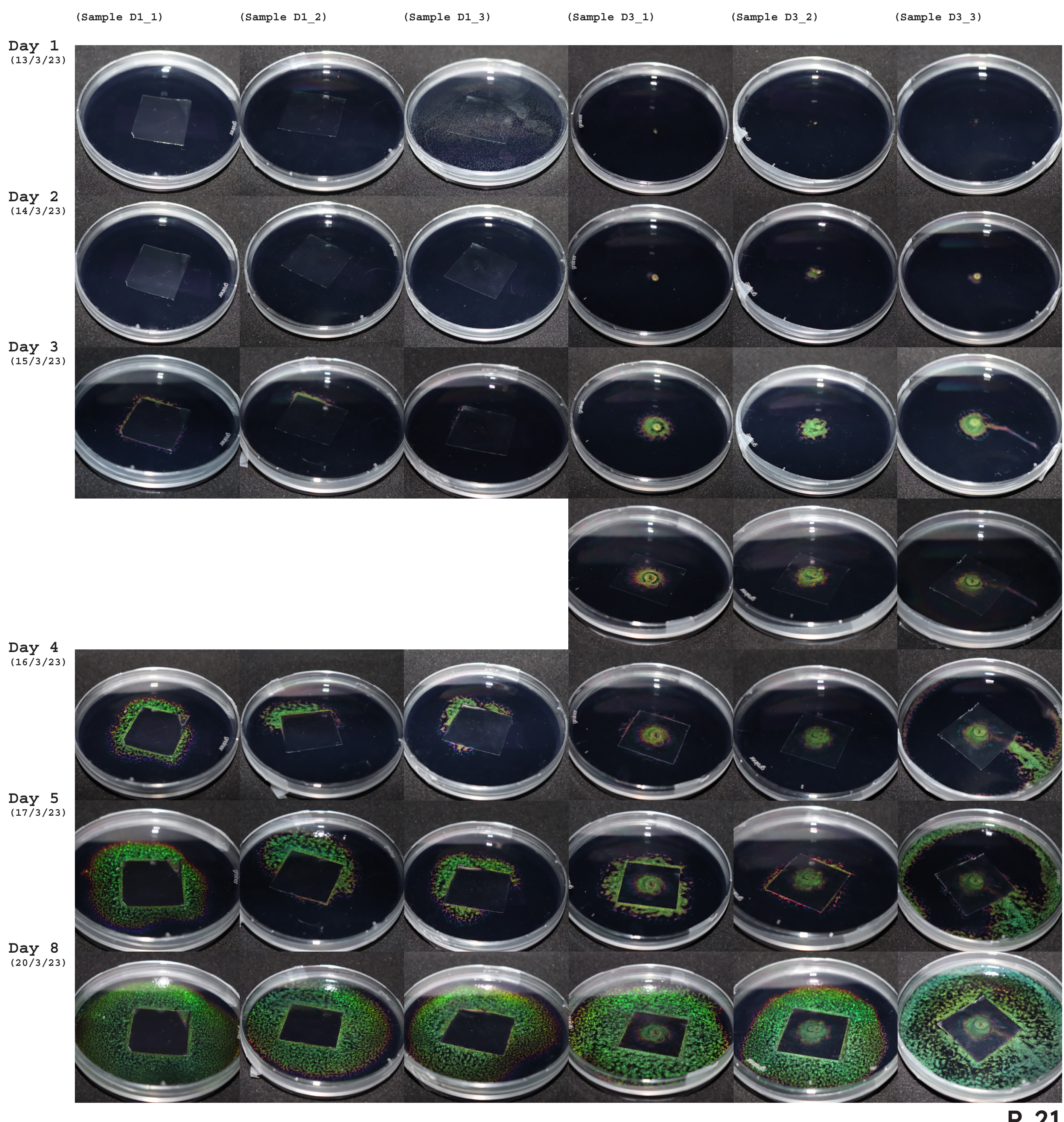


A.6.1 / NatureFlex

Top Layer (Bio4Life Material)

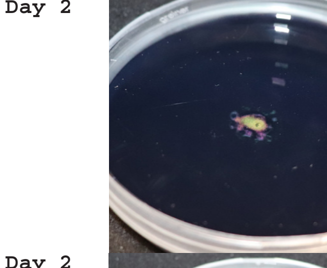


A.6.2 / PLA
Top Layer (Bio4Life Material)



A.6.3 / Silicone

Top Layer



Silicone

Day 2

Day 2

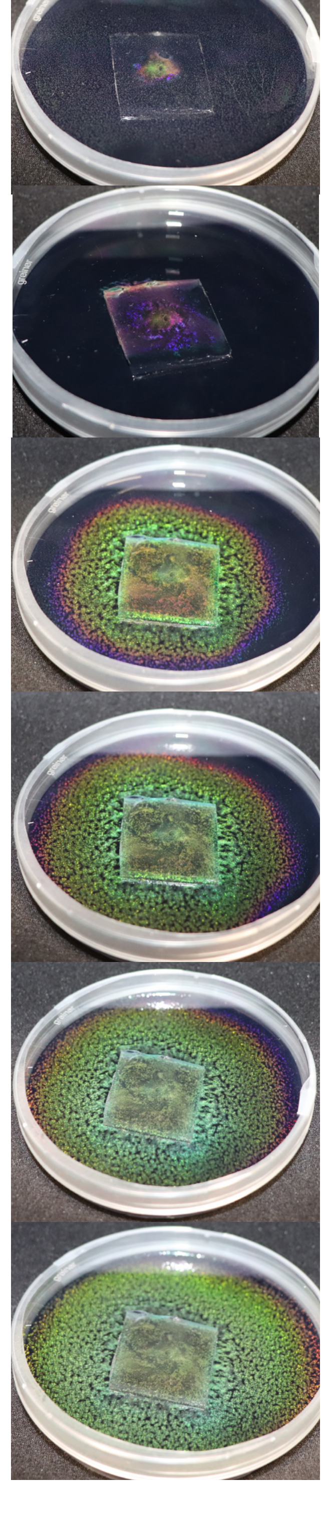
Day 3

Day 6

Day 7

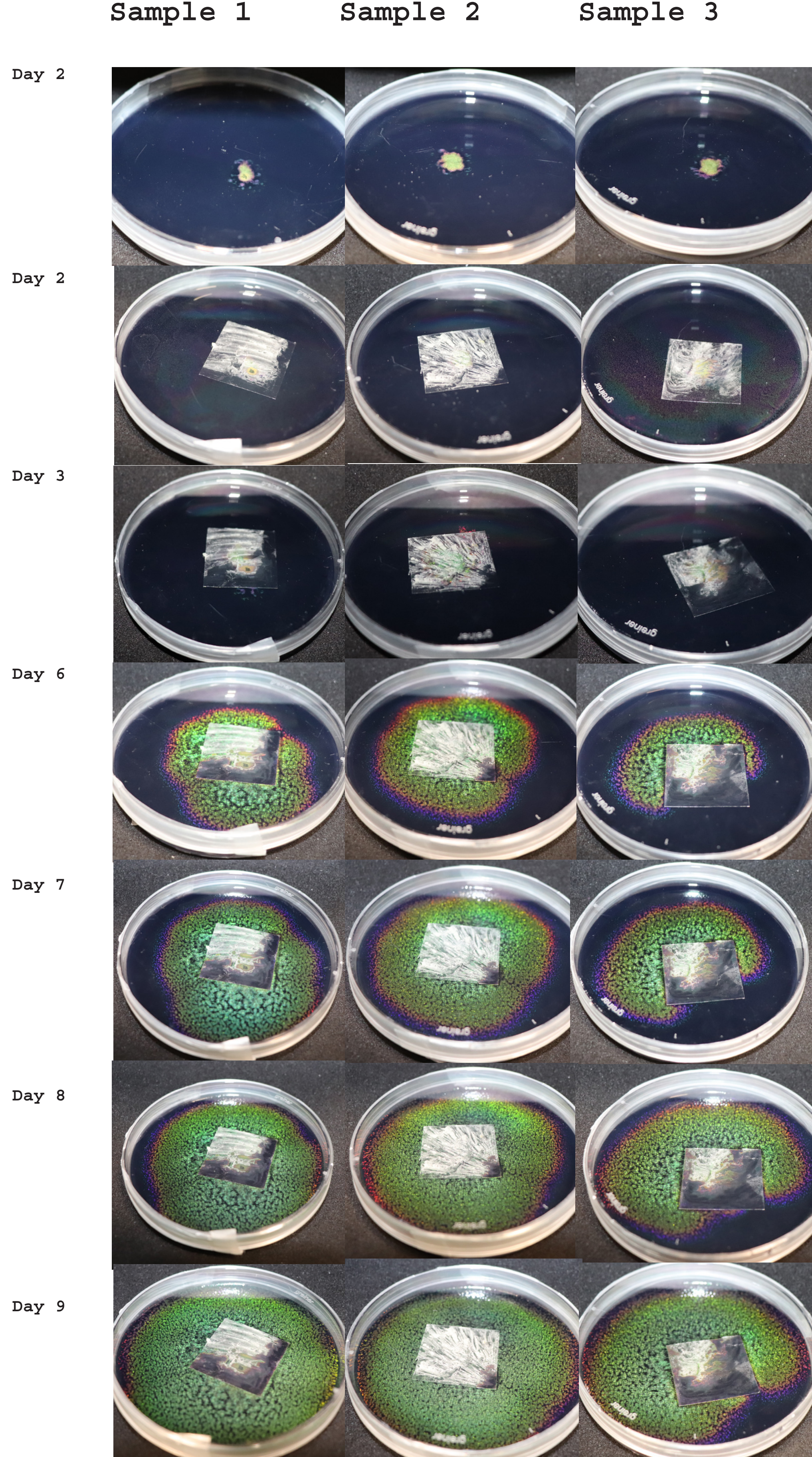
Day 8

Day 9



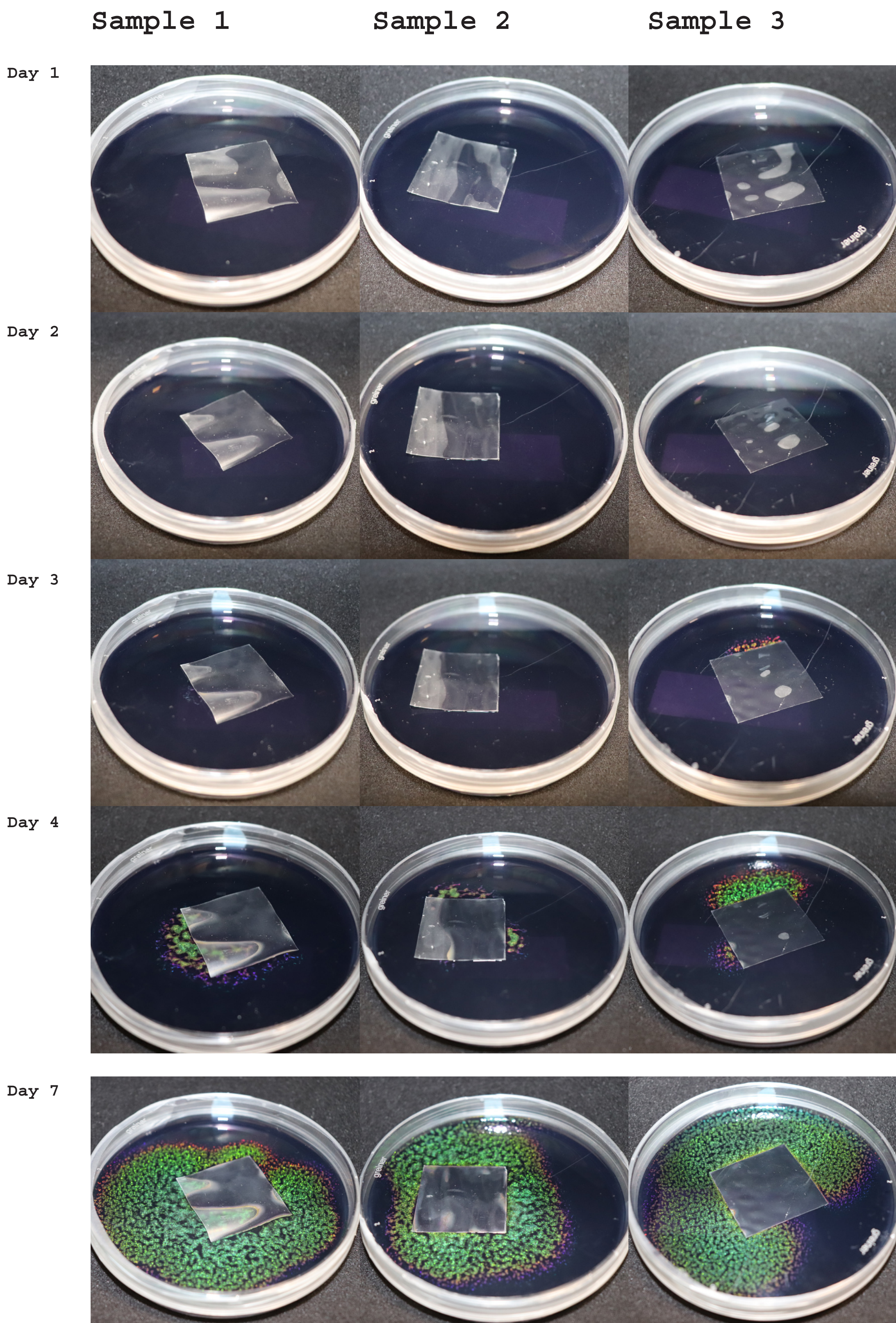
A.6.4 / Beeswax (1)

Top Layer



A.6.5 / Beeswax (2)

Top Layer



Bio Silicone Recipe

Ingredients:

48 gr gelatine
48 gr glycerine
240 gr water

Method
Since the biosilicone for this experiment needs to be made in a sterile environment, the method is slightly different than that of the recipe.

1. The first step is to weigh all of the ingredients
2. The second step is to put the gelatine into cold water for 5 minutes so that it becomes soft.
3. Then the gelatine, glycerine and water can be added together into a flask
4. This flask is then sealed with aluminium foil and closed with autoclave tape
5. This flask is then put into the autoclave for 20 min at 121 degrees celsius.
6. The flask was then put onto a heater with a magnetic stirrer at 60 degrees celsius for 25 minutes. This was done in the laminar flow cabinet
7. After 25 minutes, the liquid was pipeted in various amounts in petridishes
8. After letting the liquid cool down a bit the petri dishes were closed and sealed of with parafilm

Figure 1 - Bio Silicone

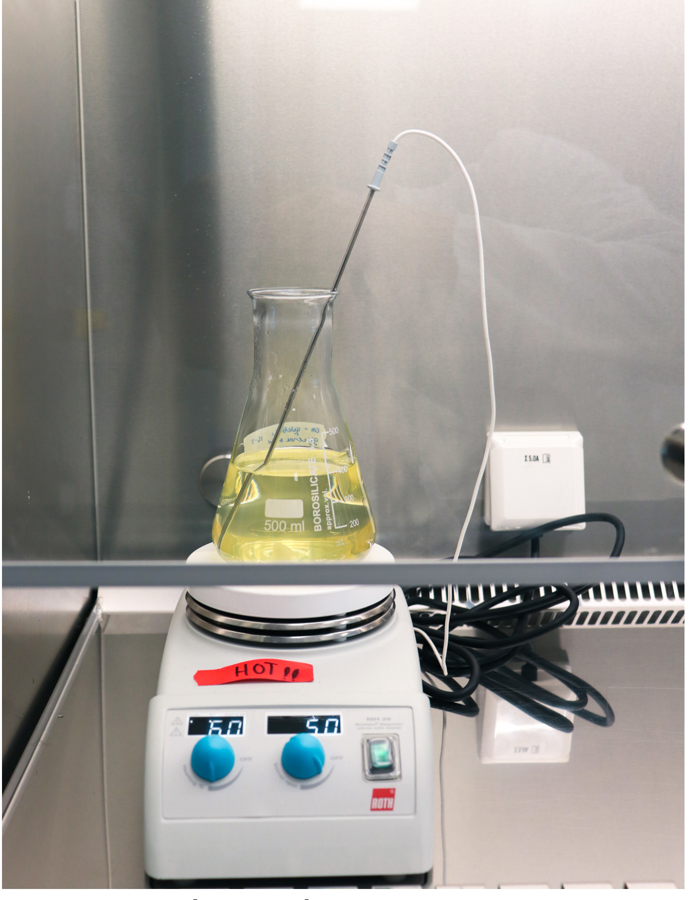
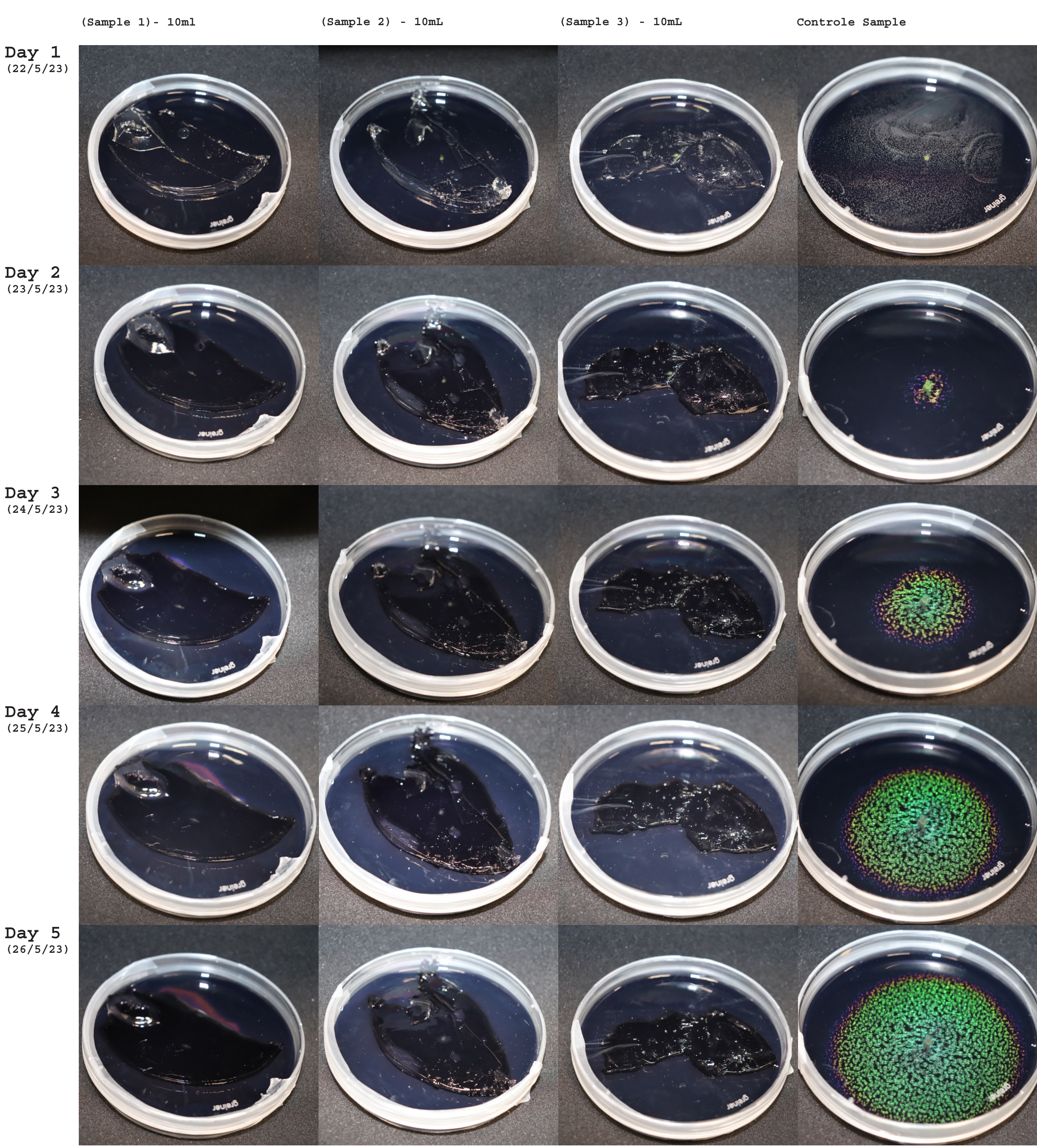


Figure 2 - Bio Silicone on heater

A.6.6 / BioSilicone

Second Try (Sterile)



A.6.7 Agar Foil
Top Layer

Agar Foil Recipe

Ingredients:

5 gr Agar
15 gr Glycerine
250 gr Water

Method
Since the agar foil for this experiment needs to be made in a sterile environment, the method is slightly different than that of the recipe.

1. The first step is to weigh all of the ingredients
2. Then the agar, glycerine and water can be added together into a bottle
3. This bottle is then closed and marked with autoclave tape
4. This bottle is then put into the autoclave for 20 min at 121 degrees celsius.
5. It was then put onto a heater with a magnetic stirrer at 60 degrees celsius for 40 minutes. This was done in the laminar flow cabinet
7. After 40 minutes, the liquid was pipeted in various amounts in petridishes
8. After letting the liquid cool down a bit the petri dishes were closed and sealed of with parafilm

Figure 1 - Agar Foil

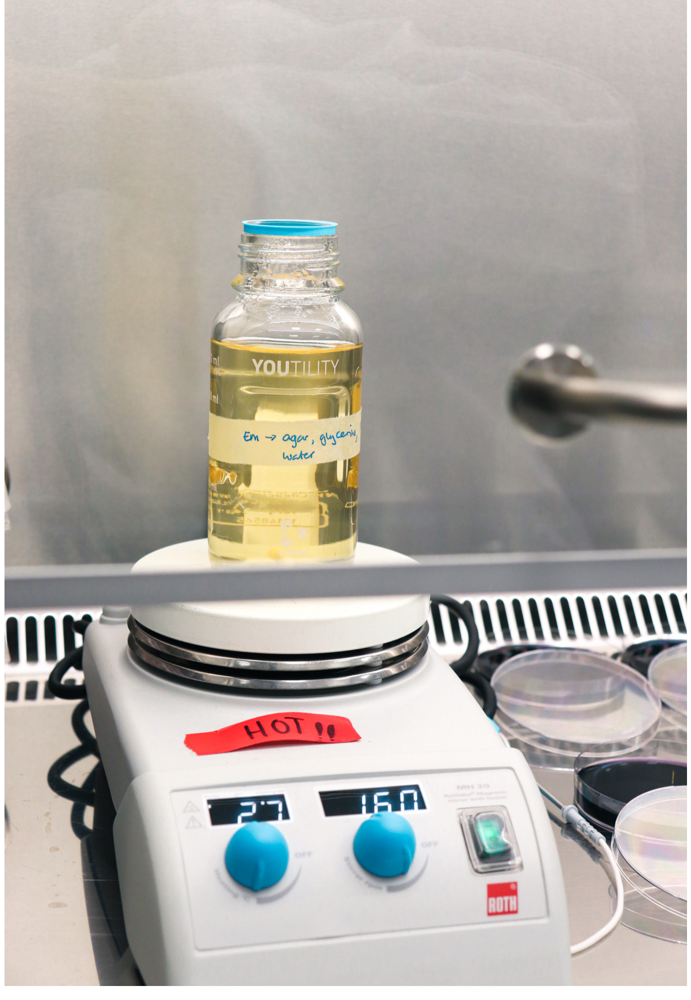
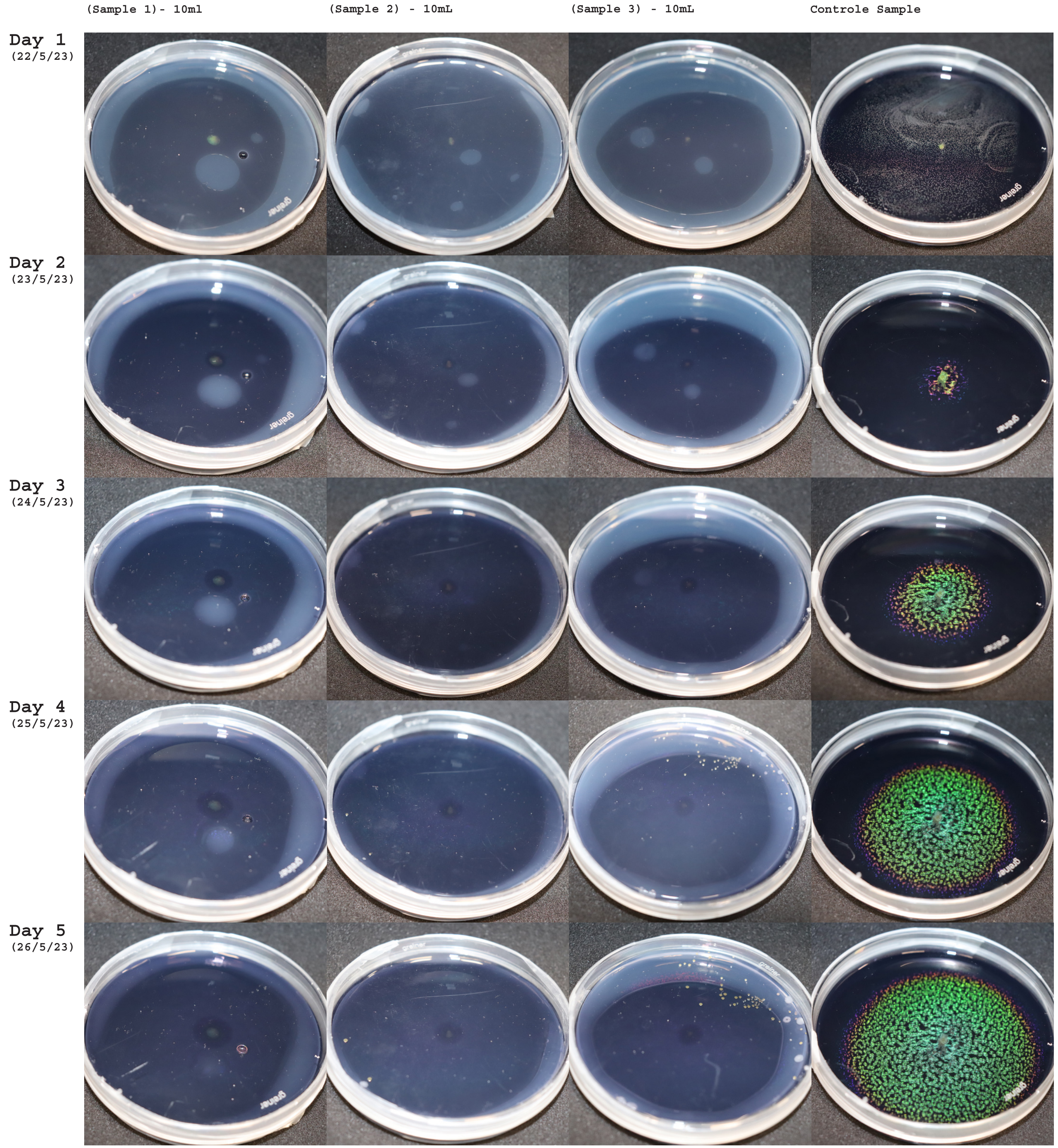


Figure 2 - Agar foil mixture on heater

A.6.7 / Agar Foil

Placed on top of Flavobacteria

Agar foil was made in a sterile environment, then let cured for 6 days and placed on top of inculated MAR medium.



A.6.7 / Agar Foil

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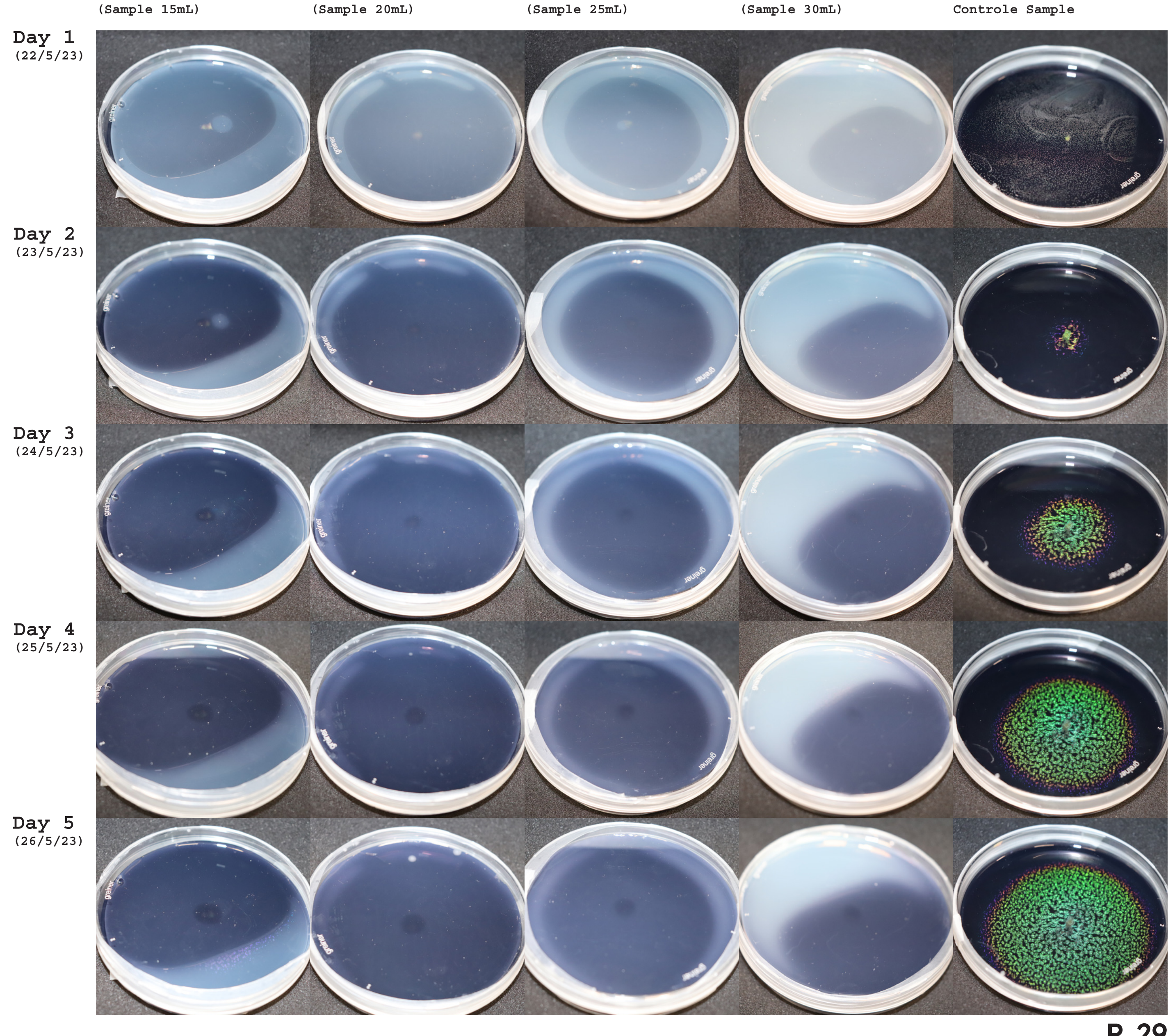


A.6.8 / Headspace
Agar foil and PLA

Sample 1 | Sample 2 | Sample 3 | Controle Sample

Day 1
(30/5/23)

Day 2
(31/5/23)

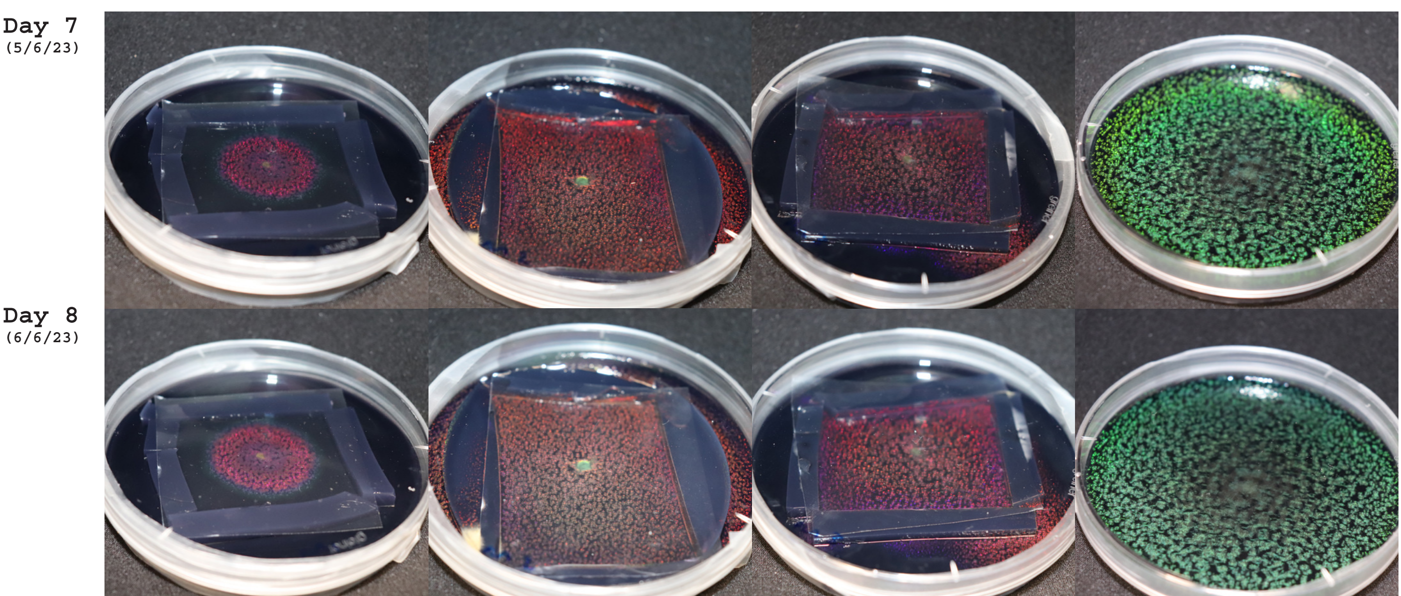
Day 3
(1/6/23)

Day 4
(2/6/23)

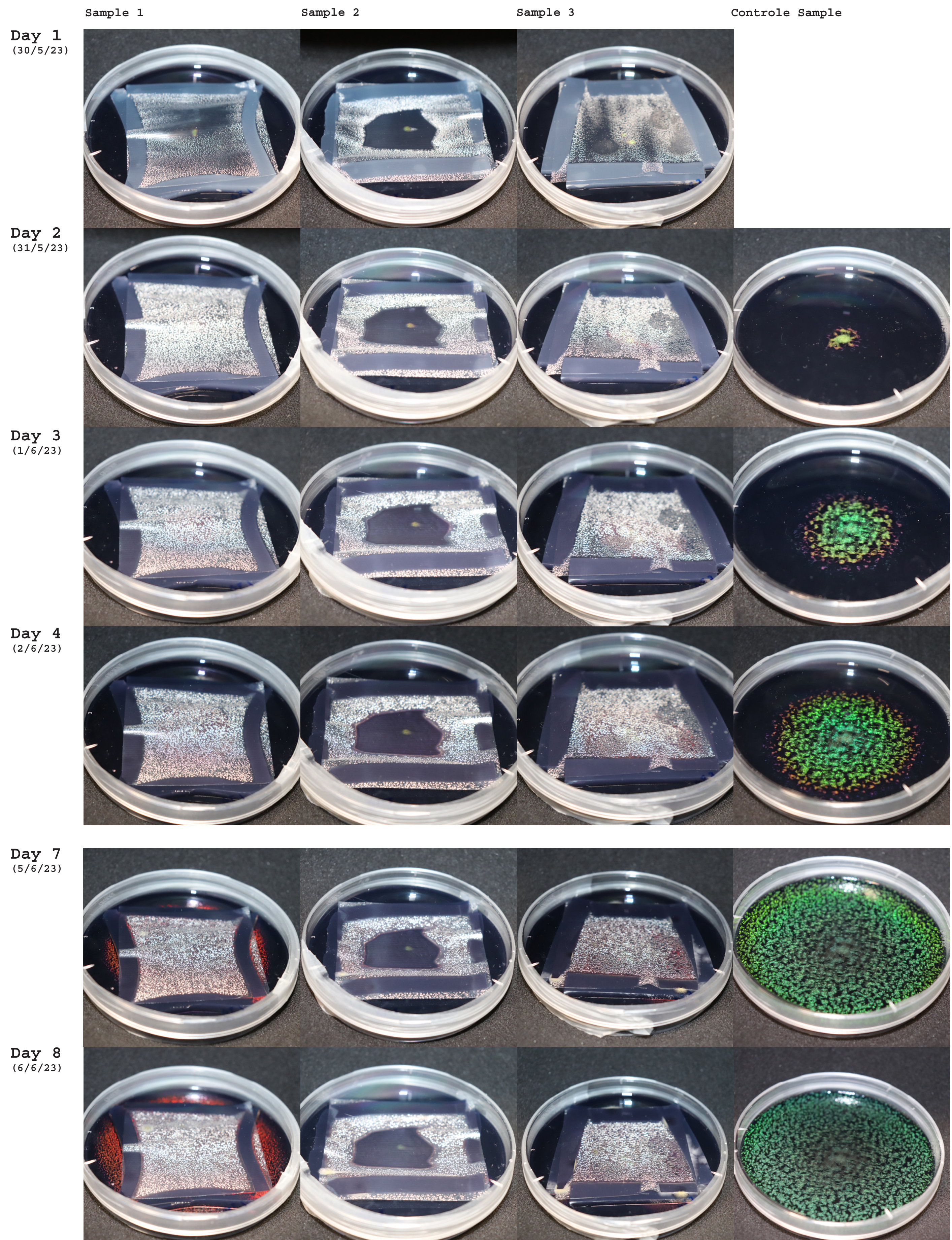
Fridge

Day 7
(5/6/23)

Day 8
(6/6/23)



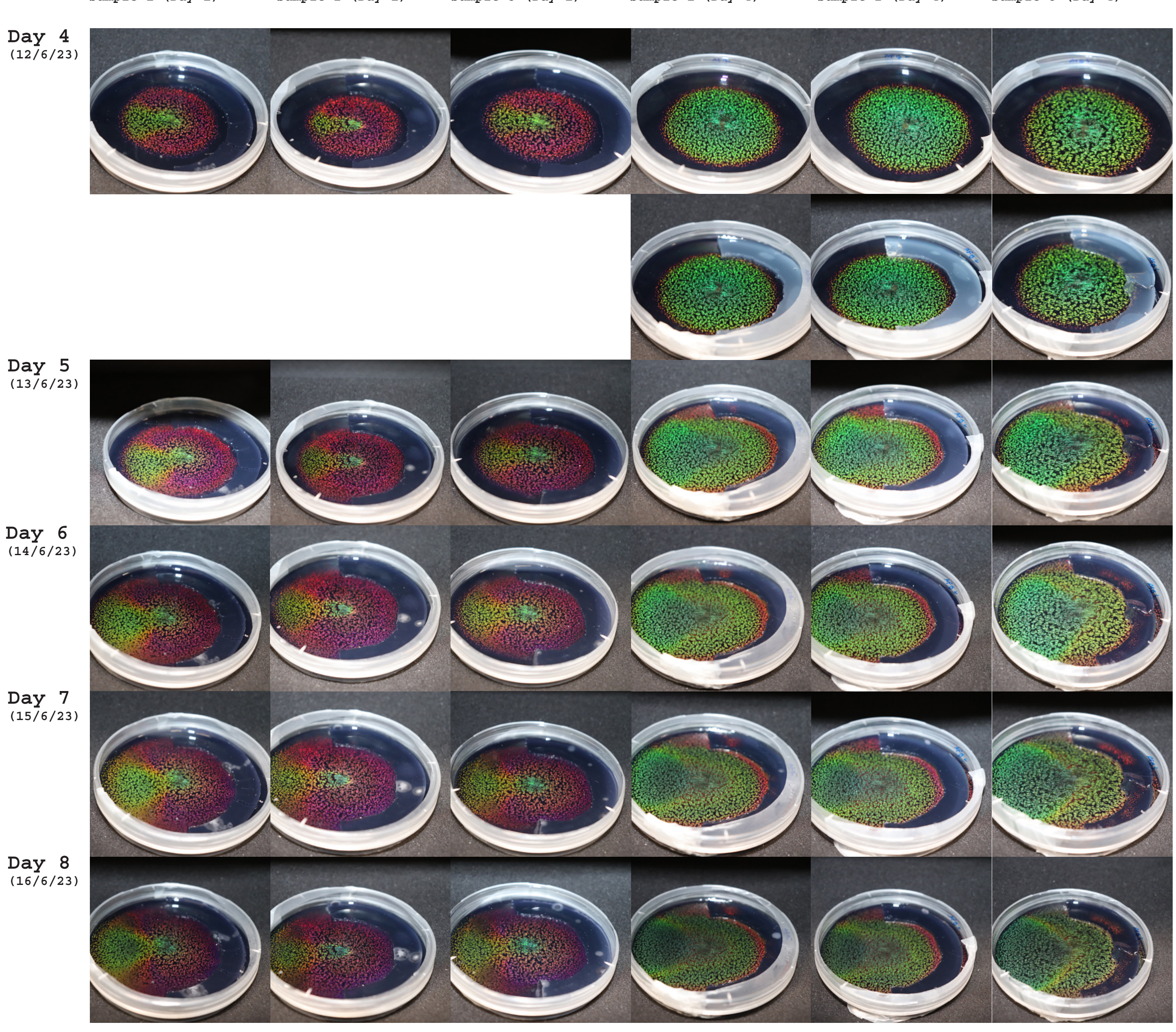
A.6.8 / Headspace
Agar foil and NatureFlex



A.6.9 / Glycerol

Influence of a piece of agar foil on *C. lytica* Colony

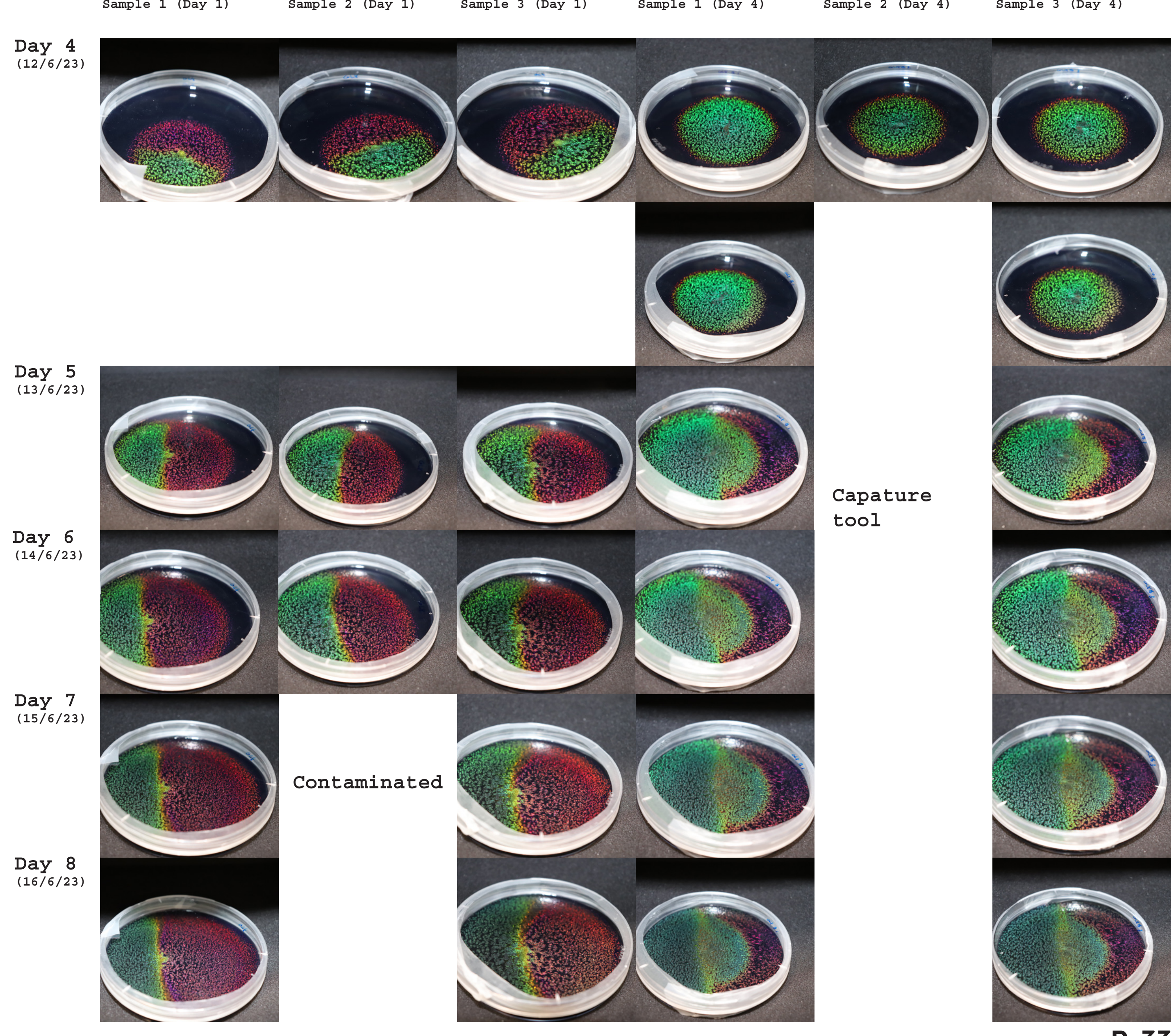
In order to test the hypothesis that agar foil was responsible for the red iridescence preceived from the structural colour of Flavobacteria in the physical headspace experiments, agar foil was placed next to a *C. lytica* colony to observe the behaviour. Agar foil was placed directly after inoculation next to the colony (Sample 1-3 day 1), or after 4 days of colony growth (Sample 1-3 day 4).



A.6.9 / Glycerol

Influence of a drop of glycerol on *C. lytica* Colony

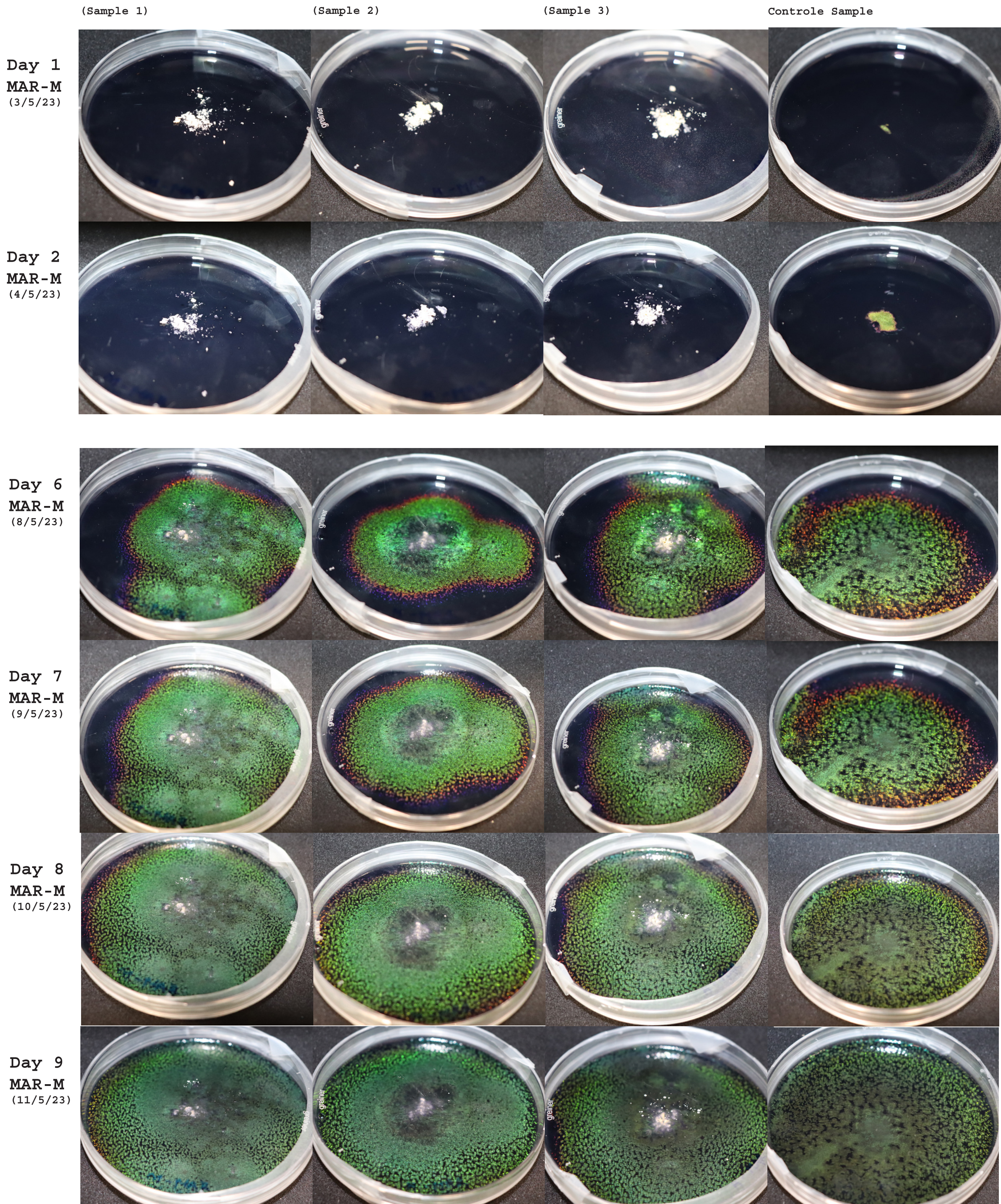
After having concluded that agar foil was indeed responsible for the red iridescence in the *C. lytica* colony, further research was done to find out which component of the agar foil was responsible. Glycerol was suspected to be the ingredient responsible for the red iridescence. Therefore a drop of glycerol (0,5 mL) was was placed directly after inoculation next to the colony (Sample 1-3 day 1), or after 4 days of colony growth (Sample 1-3 day 4).



A.7.1 / Freeze Drying 1

10% Milk Powder + Liquid Medium

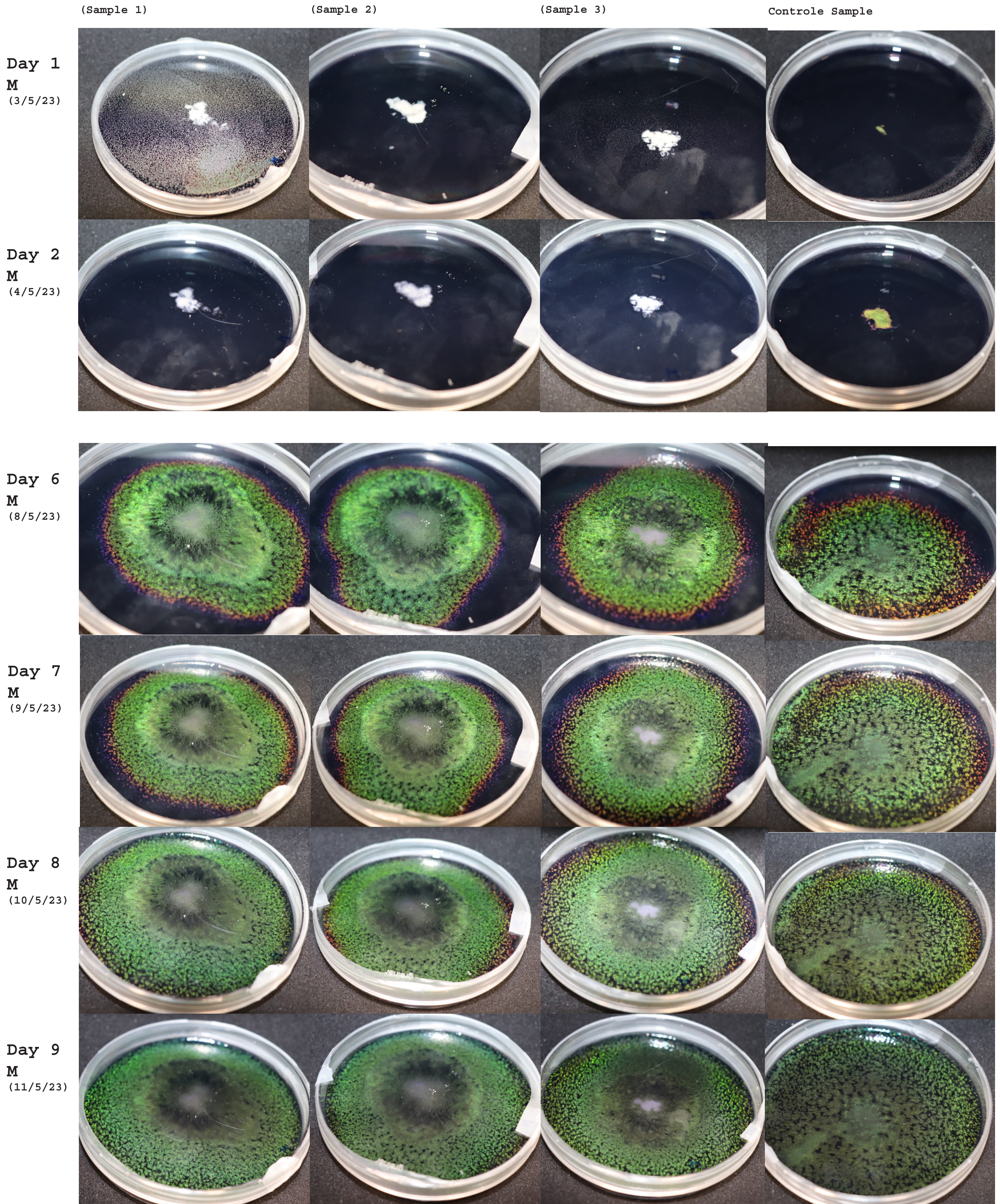
Centrifuged flavobacteria + 1,8mL 10% Milk powder and liquid medium solution, freeze dried for 24H. Then approximately 1/3 of this freeze dried substance inoculated per petri dish with MAR medium and kept in standard conditions.



A.7.1 / Freeze Drying 1

10% Milk Powder

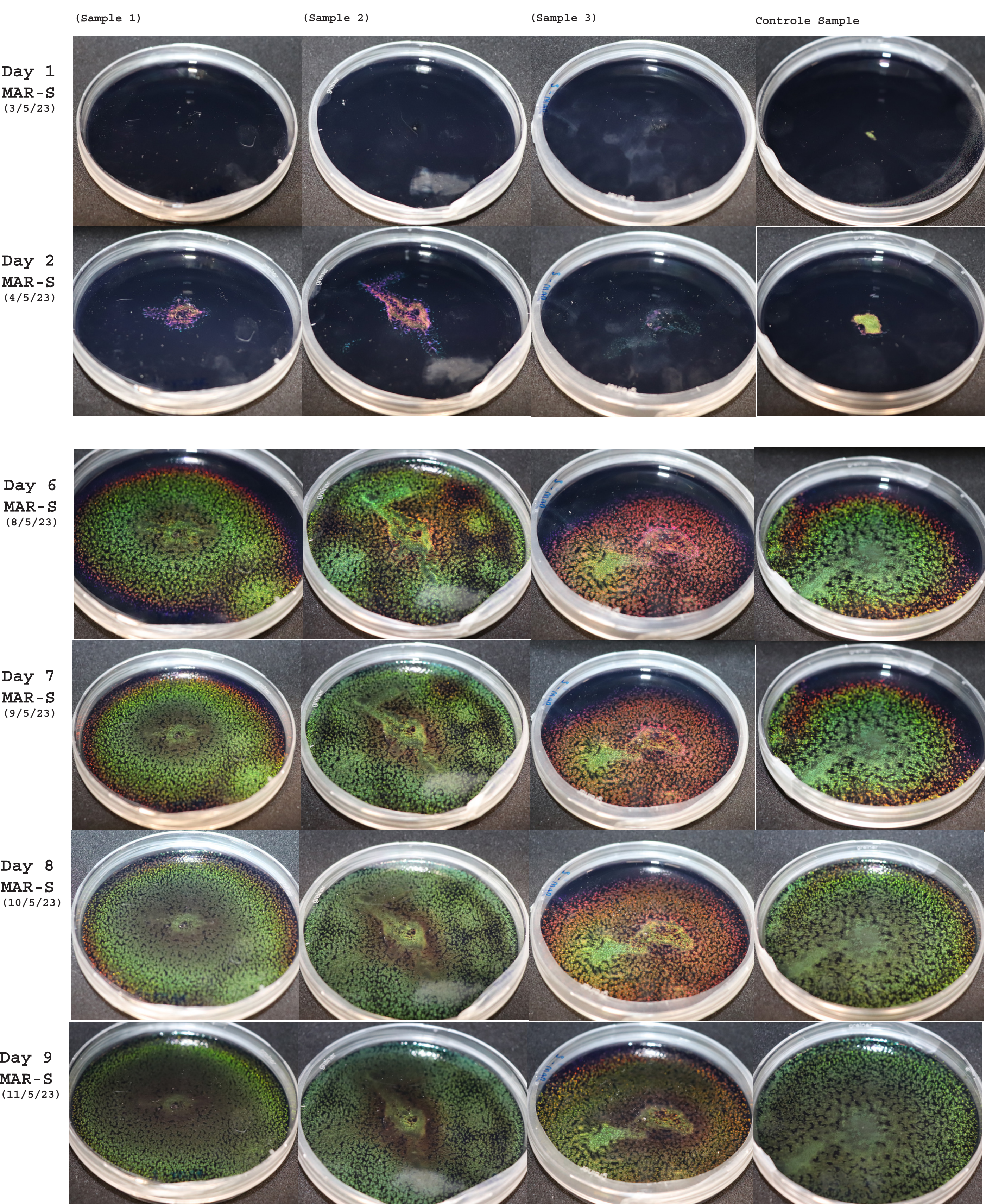
Centrifuged flavobacteria + 1,8mL 10% Milk powder solution, freeze dried for 24H. Then approximately 1/3 of this freeze dried substance inoculated per petri dish with MAR medium and kept in standard conditions.



A.7.1 / Freeze Drying 1

10% Sucrose + Liquid Medium

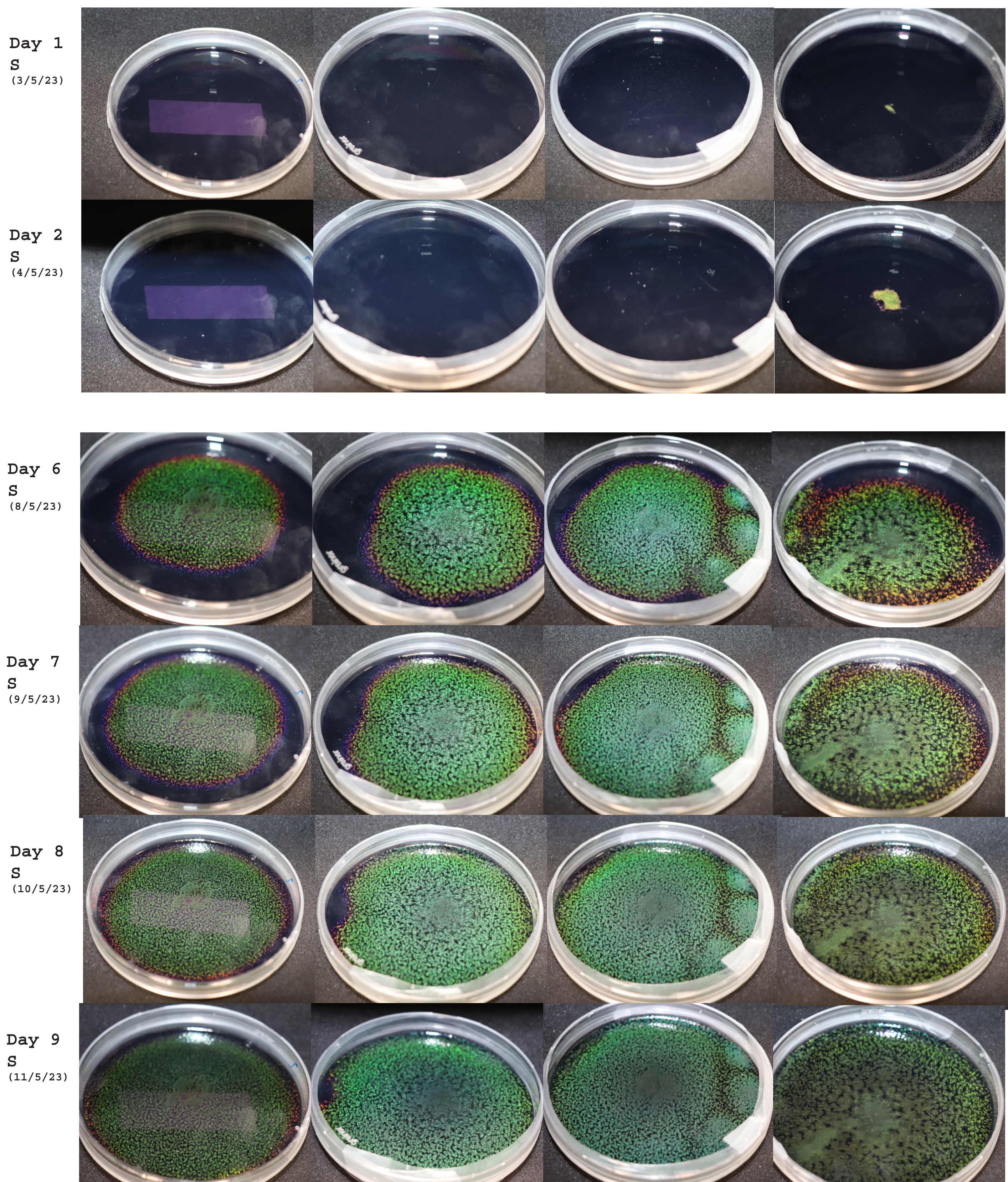
Centrifuged flavobacteria + 1,8mL 10% Sucrose and liquid medium solution, freeze dried for 24H. Then approximately 1/3 of this freeze dried substance inoculated per petri dish with MAR medium and kept in standard conditions.



A.7.1 / Freeze Drying 1

10% Sucrose

Centrifuged flavobacteria + 1,8mL 10% Sucrose solution, freeze dried for 24H. Then approximately 1/3 of this freeze dried substance inoculated per petri dish with MAR medium and kept in standard conditions.



A.7.2 / Freeze Drying 2 (Frozen)

10% Milk Powder + Liquid Medium

Centrifuged flavobacteria + 1,8mL 10% Milk powder and liquid medium solution frozen in the -80 freezer, then freeze dried for 24H. Then approximately 1/3 of this freeze dried substance inoculated per petri dish with MAR medium and kept in standard conditions.

(Sample 1) (Sample 2) (Sample 3) Controle Sample

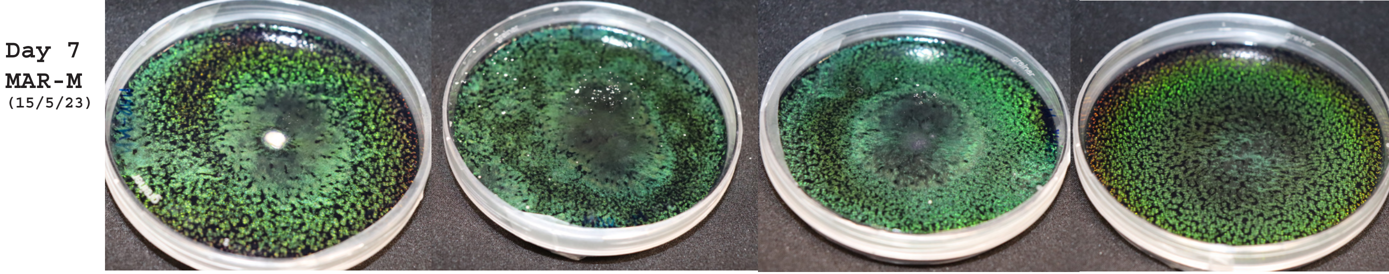
Day 1
MAR-M
(9/5/23)

Day 2
MAR-M
(10/5/23)

Day 3
MAR-M
(11/5/23)

Day 4
MAR-M
(12/5/23)

Day 7
MAR-M
(15/5/23)



A.7.2 / Freeze Drying 2 (Frozen)

10% Milk Powder

Centrifuged flavobacteria + 1,8mL 10% Milk powder solution frozen in the -80 freezer, then freeze dried for 24H. Then approximately 1/3 of this freeze dried substance inoculated per petri dish with MAR medium and kept in standard conditions.

(Sample 1) (Sample 2) (Sample 3) Controle Sample

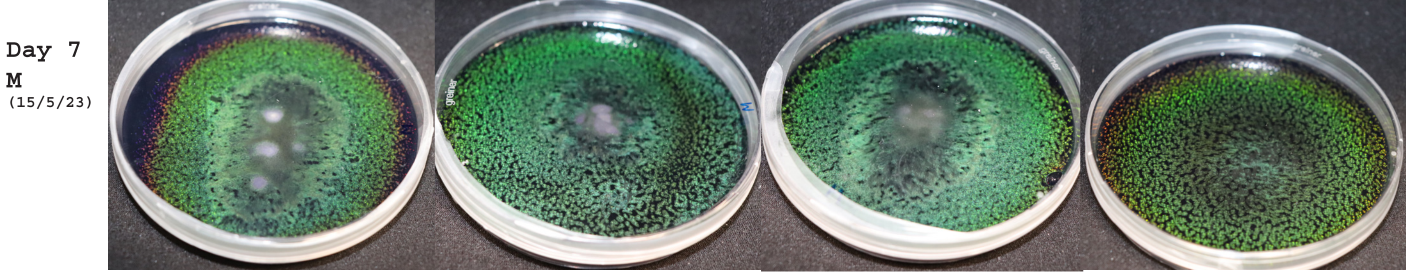
Day 1
M
(9/5/23)

Day 2
M
(10/5/23)

Day 3
M
(11/5/23)

Day 4
M
(12/5/23)

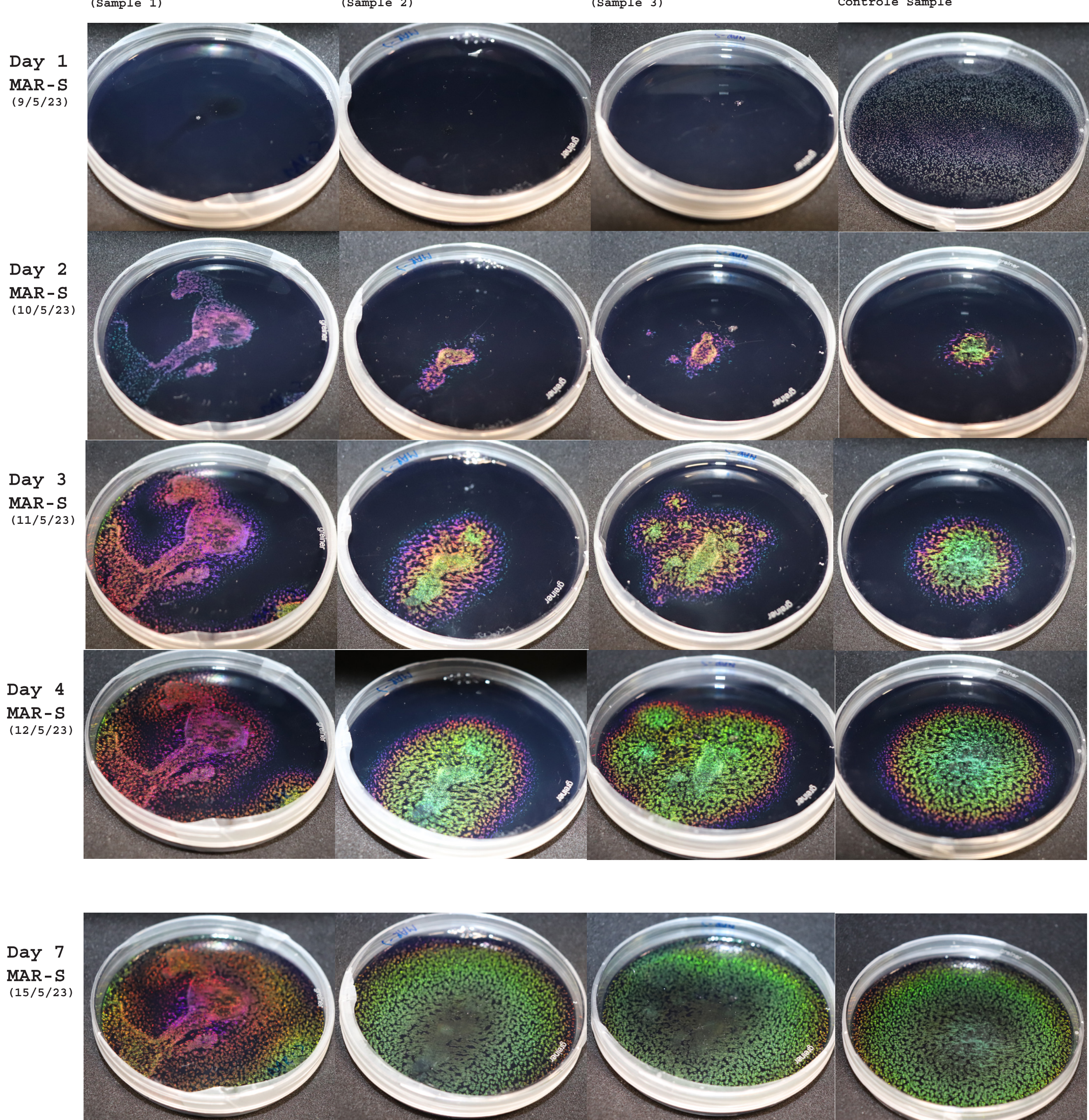
Day 7
M
(15/5/23)



A.7.2 / Freeze Drying 2 (Frozen)

10% Sucrose + Liquid Medium

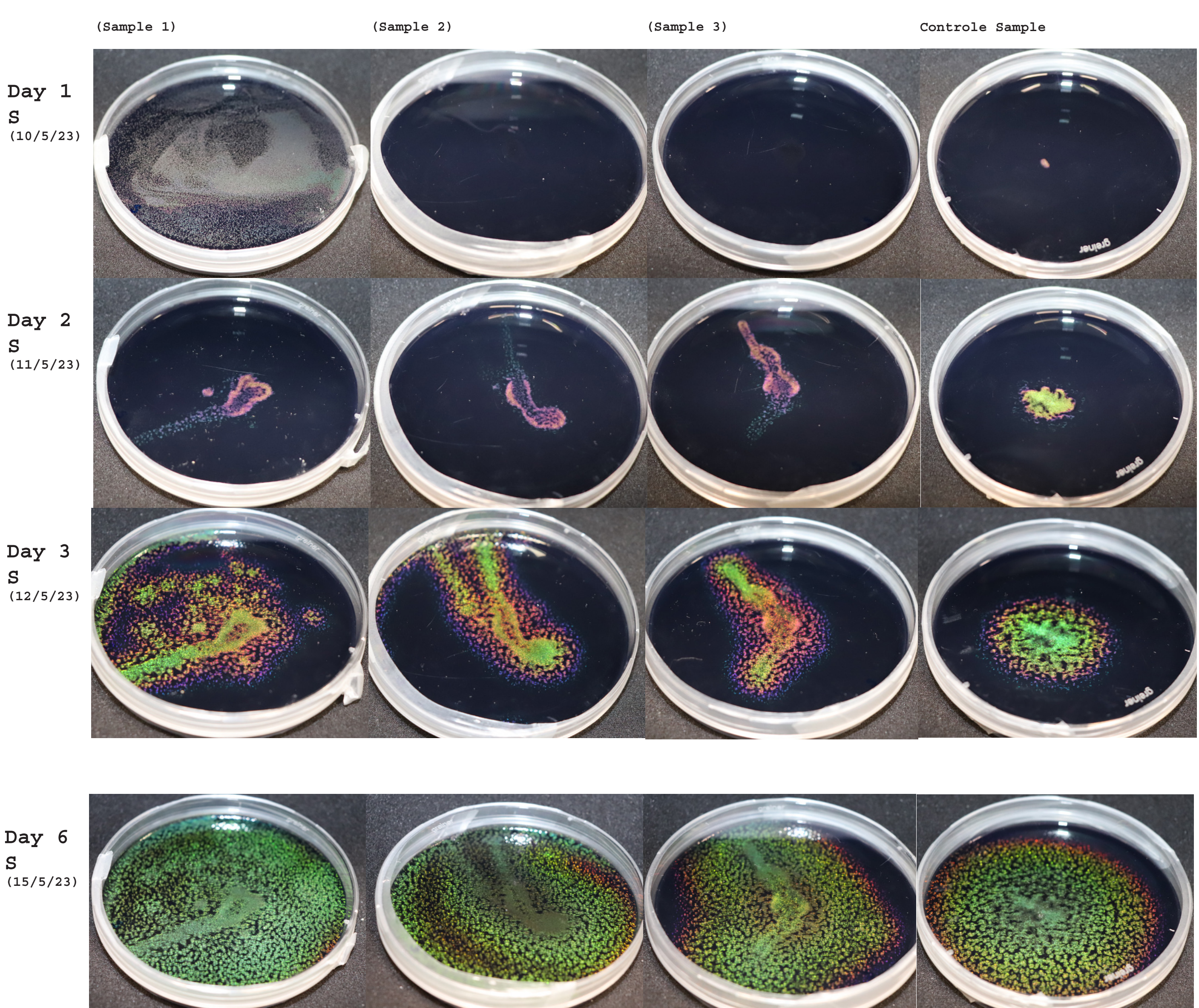
Centrifuged flavobacteria + 1,8mL 10% Sucrose and liquid medium solution frozen in the -80 freezer, then freeze dried for 24H. Then approximately 1/3 of this freeze dried substance inoculated per petri dish with MAR medium and kept in standard conditions.



A.7.2 / Freeze Drying 2 (Frozen)

10% Sucrose

Centrifuged flavobacteria + 1,8mL 10% Sucrose solution frozen in the -80 freezer, then freeze dried for 48H. Then approximately 1/3 of this freeze dried substance inoculated per petri dish with MAR medium and kept in standard conditions.



A.7.3 / Freeze Drying Flavobacteria
Protocol

Method

1. The first step of the process is to harvest your Flavobacteria from the agar plates. I have done this by sterilizing two spatula's and then scraping the Flavobacteria off of their plate. Then I transferred the bacteria to the 100 mL liquid medium.

2. Once all the Flavobacteria are harvested and transferred into the liquid medium, the flavobacteria need to be spread out evenly over the liquid medium. This is done by inserting a 25 mL pipet into the liquid medium and alternating between sucking up 15 mL and then pouring it down again. Repeat this untill there are no visbile chunks of Flavobacteria anymore.

3. Next this liquid medium with Flavobacteria is divided into 4 sterile tubes. Each tube with an amount of 22mL. We do not use 25 mL because we want to make sure that there is exactly the same amount in each tube.

4. The four tubes are then placed in the centrifuge. It is important that the weight is evenly distributed to not harm the machine. The tubes will centrifuge for 5 minutes at a speed of 4000 rpm. The accelleration is set to 9 and the decceleration to 7.

5. When taken out of the centrifuge, a clear distinction between the liquid medium and the heavier chunck of Flavobacteria in the bottom can be observed. We remove the excess liquid medium simply by pouring it out.

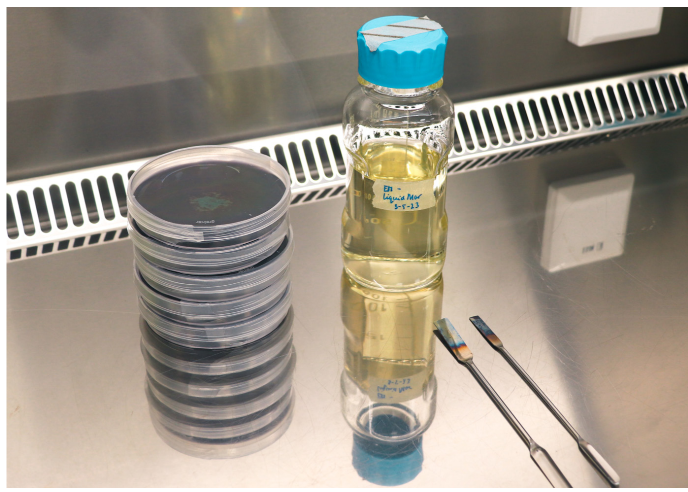
6. Once only the Flavobacteria stuck in the bottom are left in the tubes, we add 5mL (this depends on the amount of small tubes you wan to make) of the different protectants to the corresponding tubes. Each tube has a different protectant. So we have one tube with 10% milk solution, 10% milk solution + liquid medium, sucrose solution and sucrose solution + liquid medium (as mentioned before).

7. These solutions will then be mixed by vortexing.

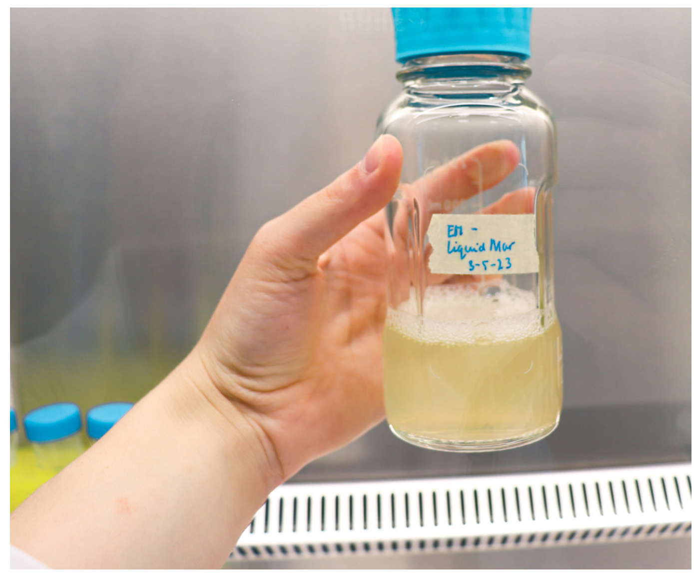
8. Next, 1,5 mL is pipeted per small tube. This results in 3 small tubes per protective agent and 12 tubes in total.

9. We then put all of these tubes into the -80 freezer. (this step is new, the first batch this was not done and all of the samples bubbled out of their tubes.)

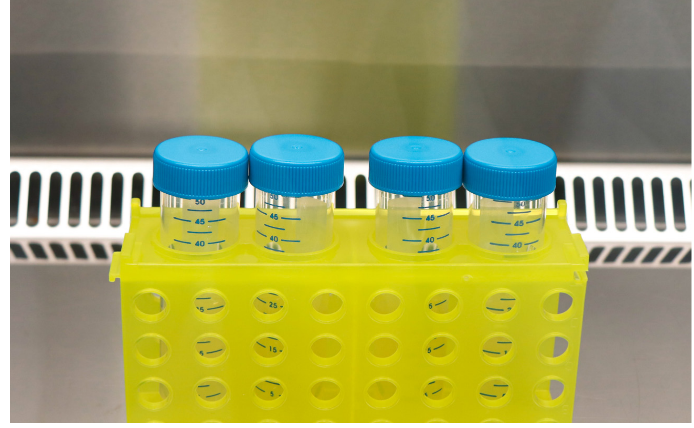
10. Freeze dry for 24/48 hours (depends on protectant)



Step 1



Step 2

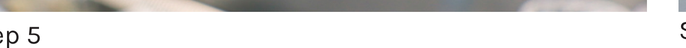


Step 3

A.7.3 / Freeze Drying Flavobacteria

Protocol

Step 4

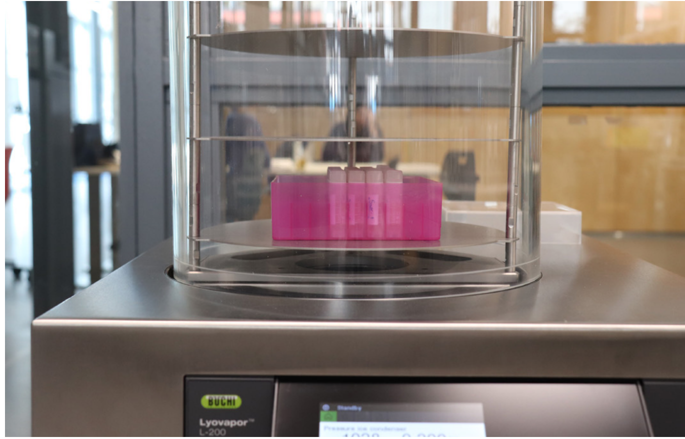


Step 5

Step 7

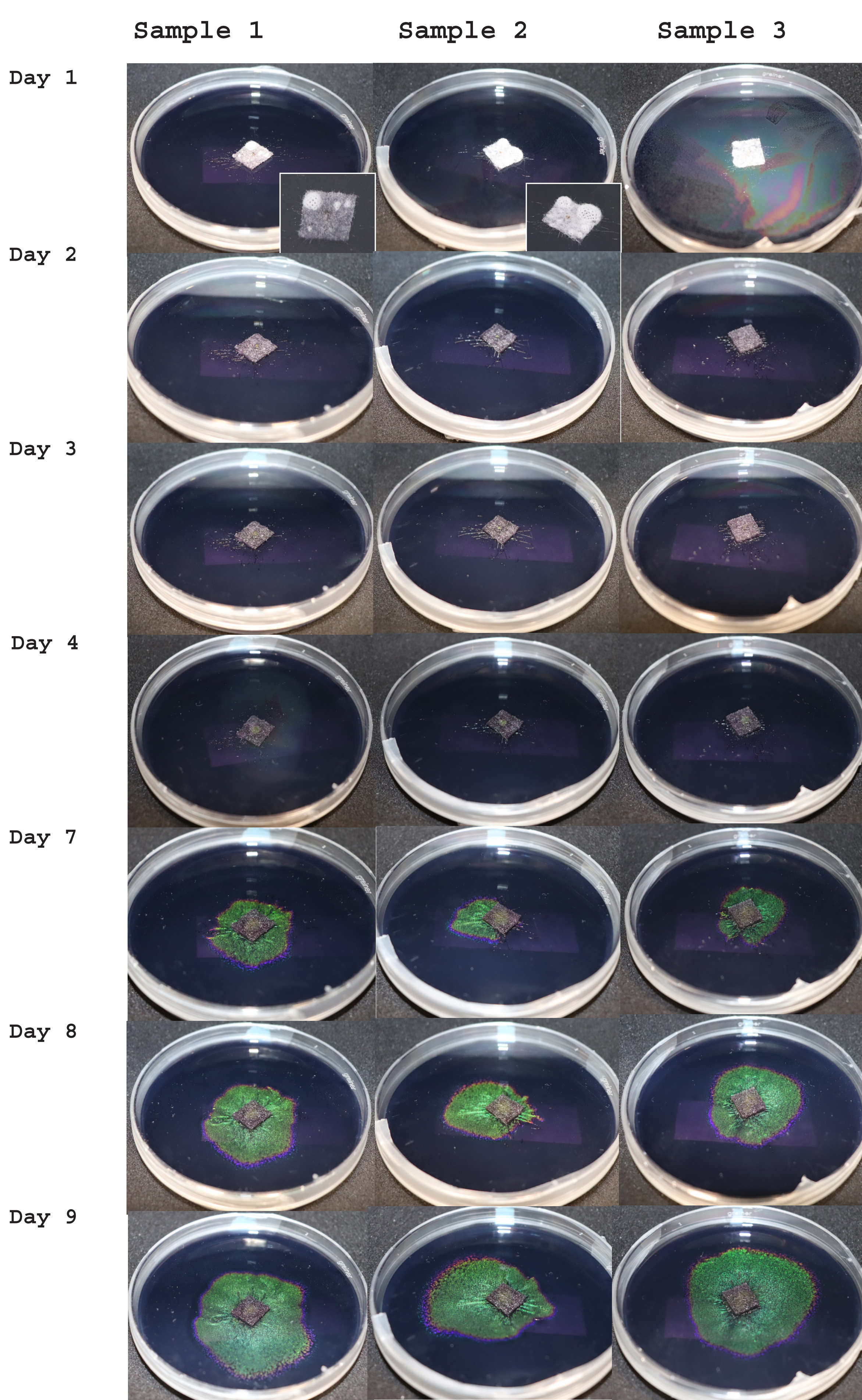
Step 8

Step 9



Step 10

A.8.1 / PVA
Alternative Medium



A.8.2 / PaperWise
Alternative Medium

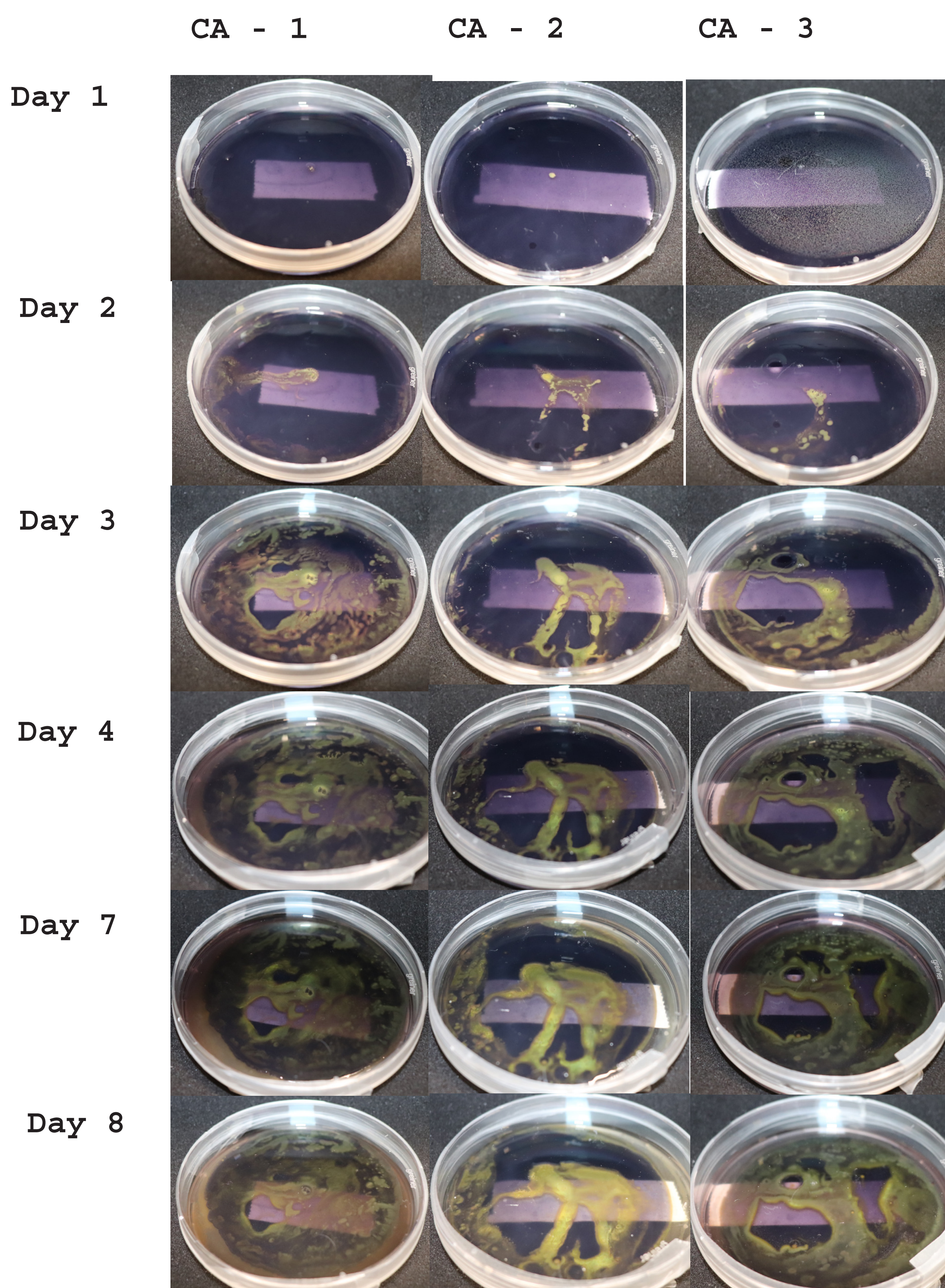
	Sample 1	Sample 2	Sample 3
Day 2			
Day 3			
Day 6			
Day 7			
Day 8			
Day 9			

A.8.3 / Calcium Alginate (Soaking)

Alternative Medium

Method

1. Prepare Agar medium without nutrients
2. Pour CalciumChloride Solution over the top
3. Remove excess liquid
4. Pour Alginate over the top
5. Remove excess liquid

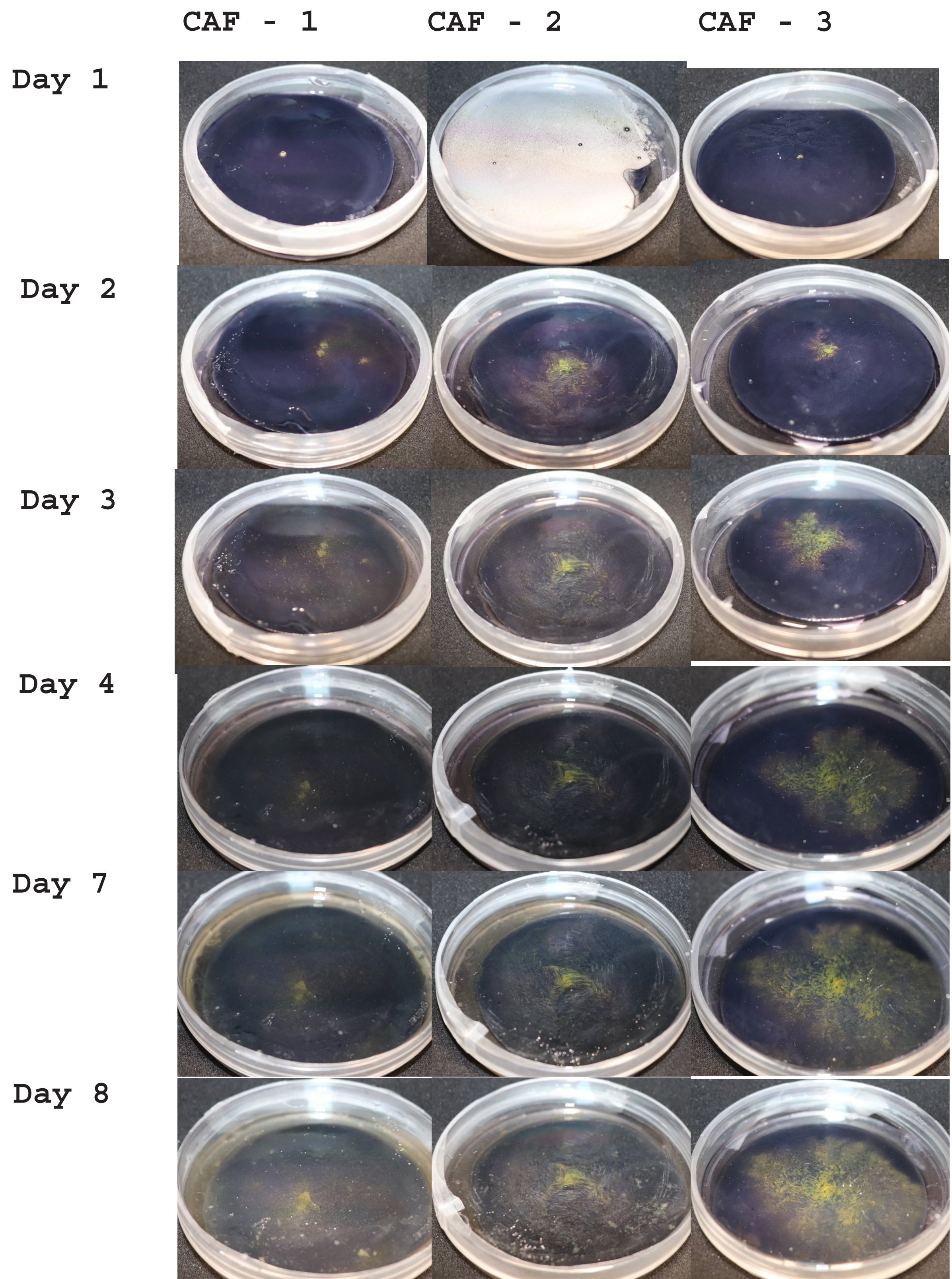


A.8.4 / Calcium Alginate (Freezing)

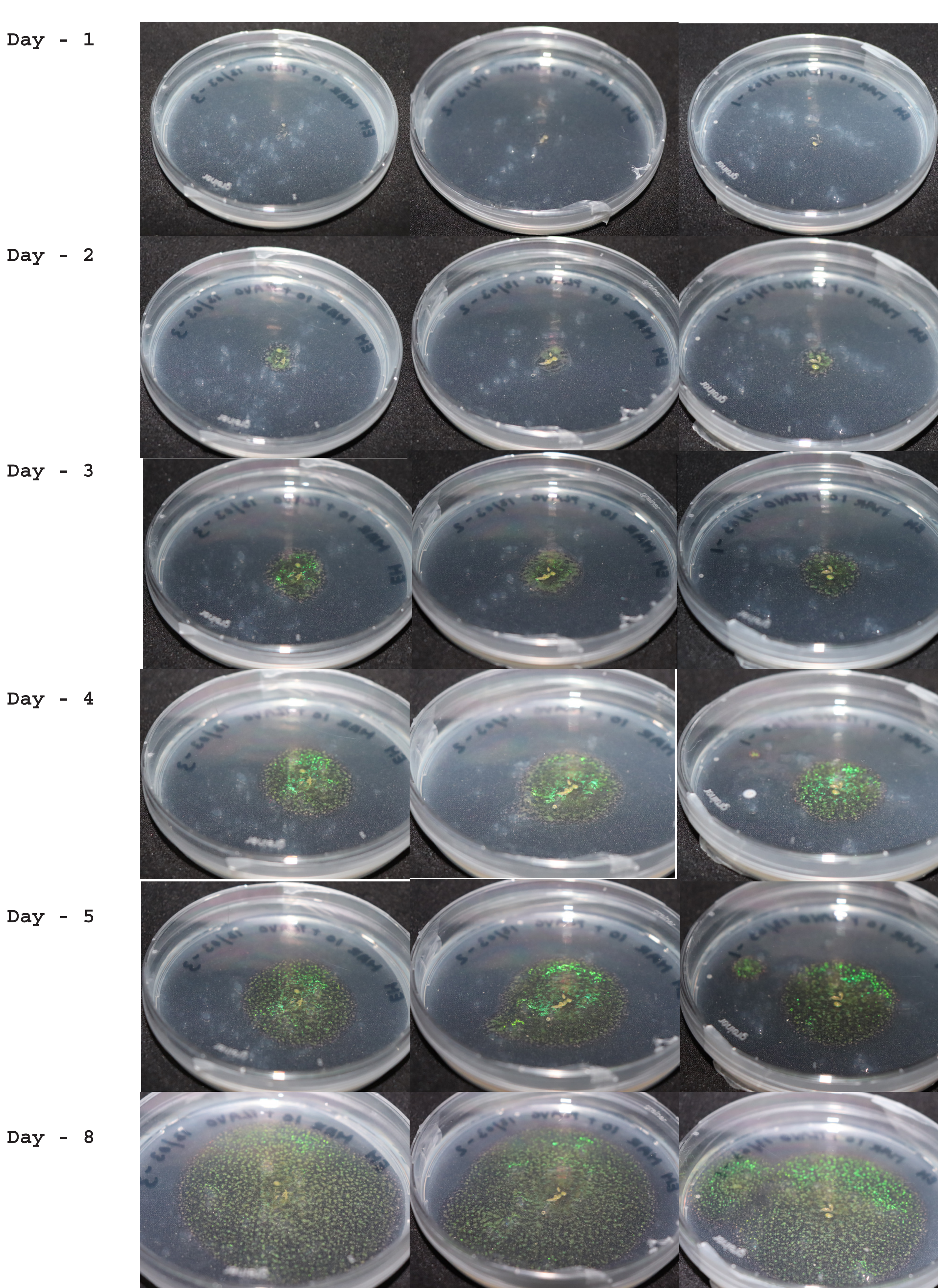
Alternative Medium

Method

1. Freeze Alginate in -20 freezer
2. Pour CalciumChloride Solution over the top
3. Remove excess liquid



A.8.5 / Medium Thickness (10mL)
Alternative Medium



A.8.5 / Medium Thickness (15mL)

Alternative Medium

	15 mL - 3	15 mL - 2	15 mL - 1
Day - 1			
Day - 2			
Day - 3			
Day - 4			
Day - 5			
Day - 8			

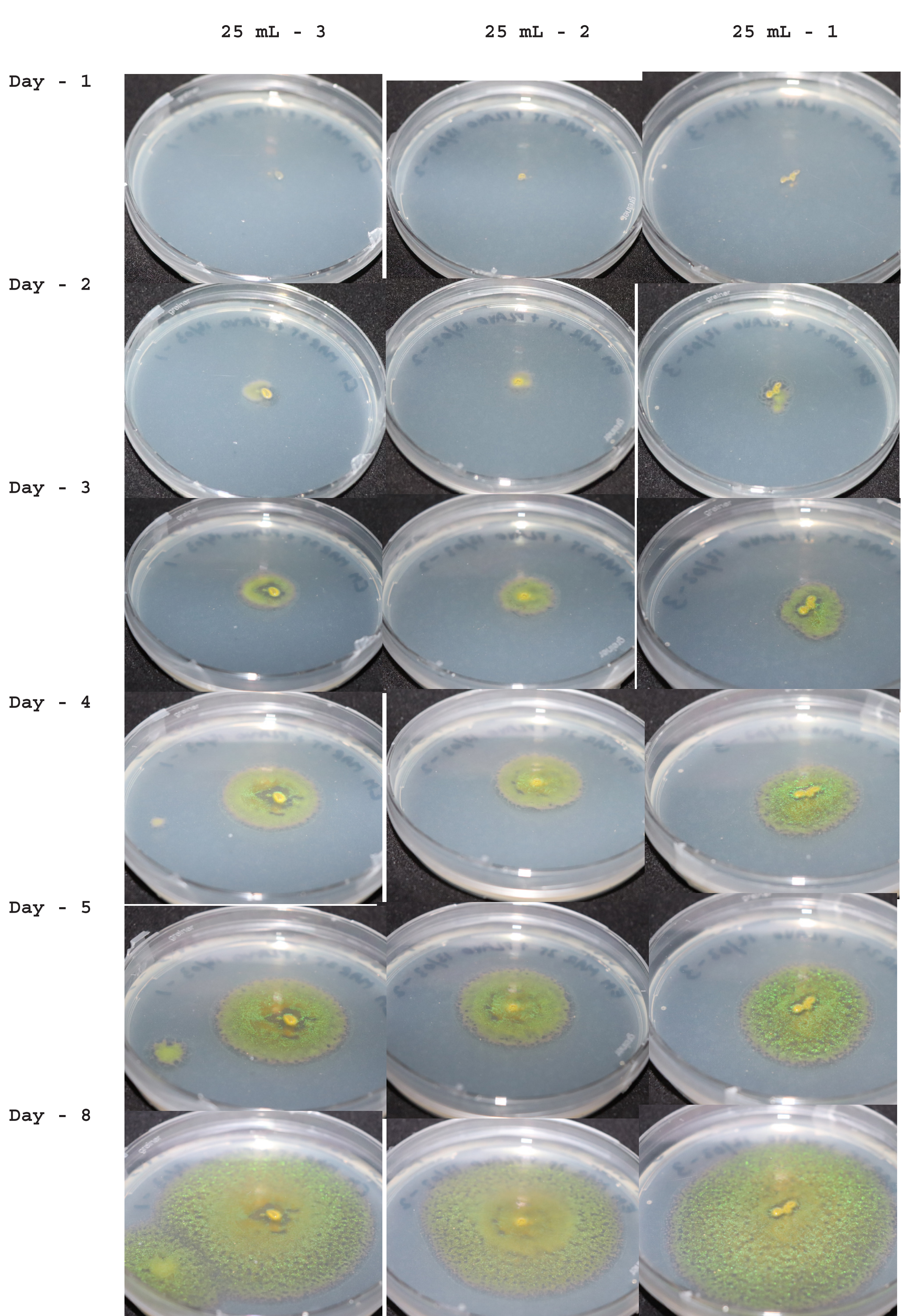
A.8.5 / Medium Thickness (20mL)

Alternative Medium

	20 mL - 3	20 mL - 2	20 mL - 1
Day - 1			
Day - 2			
Day - 3			
Day - 4			
Day - 5			
Day - 8			

A.8.5 / Medium Thickness (25mL)

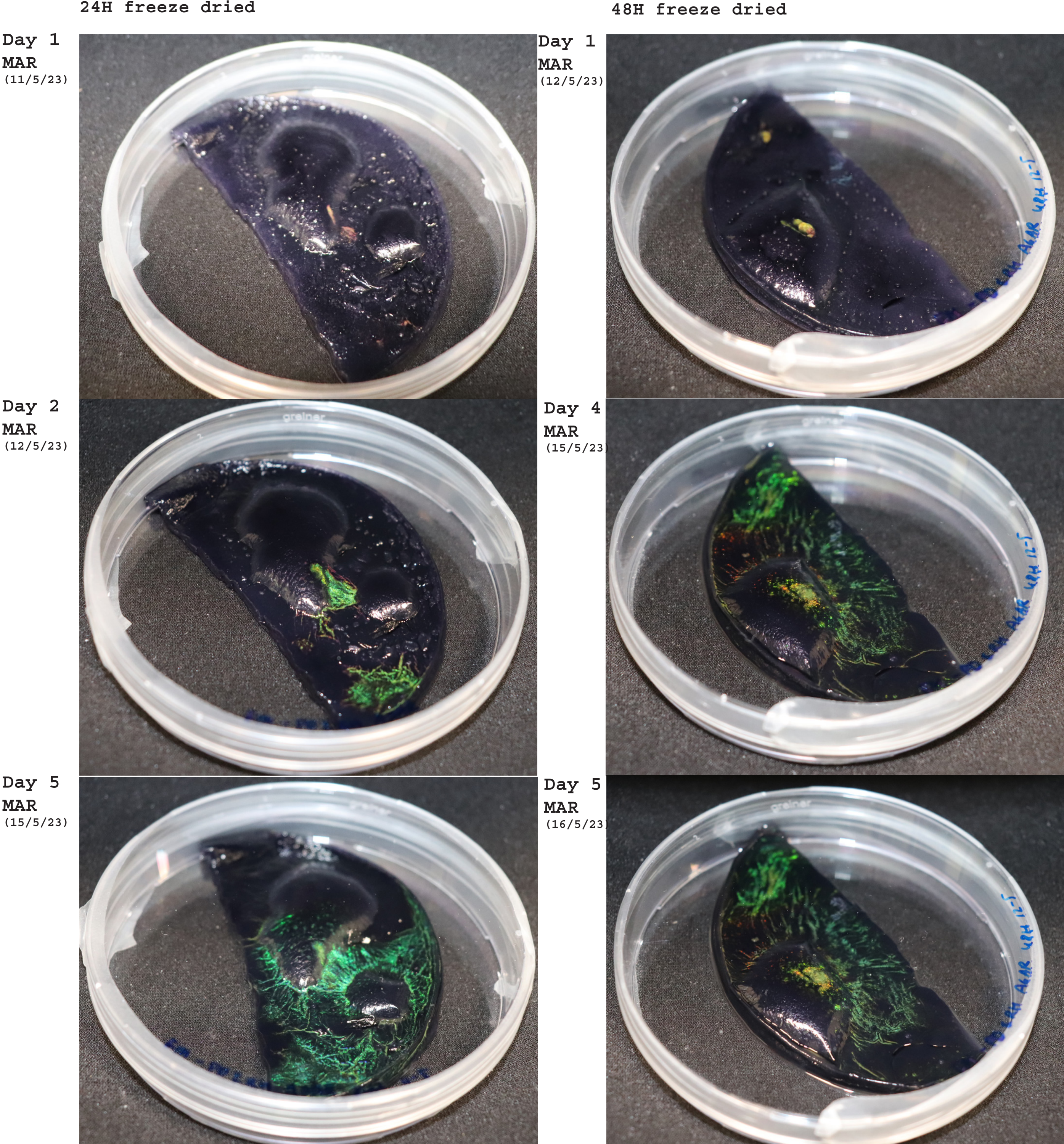
Alternative Medium



A.8.6 / Rehydated MAR Medium

Alternative Medium

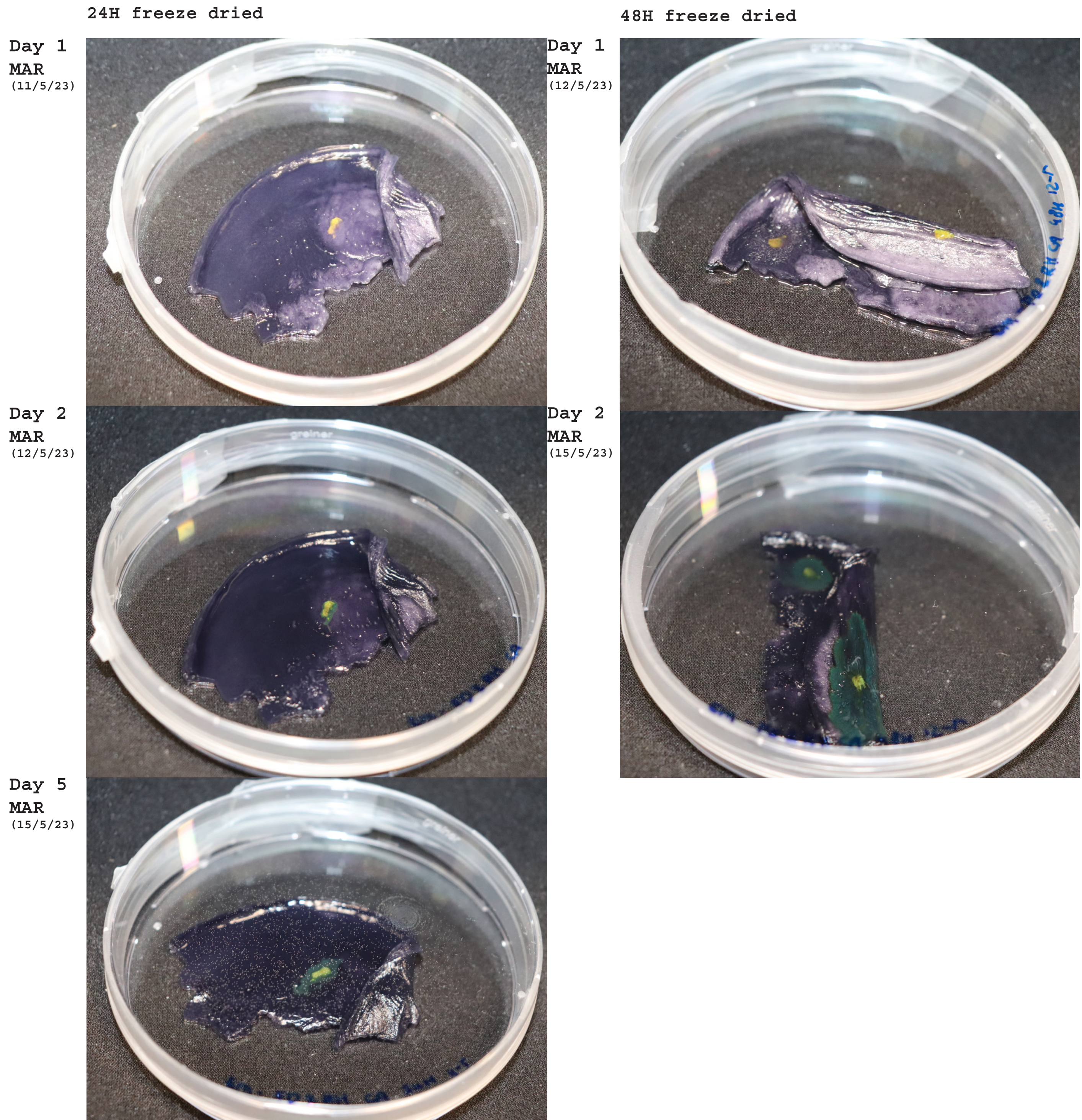
25mL MAR medium, freeze dried for 24H, then cut in half. One half rehydrated with 25mL demi water, the other half freeze dried for another 24H and then rehydrated with 25mL water. After rehydration inoculated with Flavobacteria on several places.



A.8.7 / Rehydated Calcium Alginate Medium

Alternative Medium

25mL Calcium Alginate medium, freeze dried for 24H, then cut in half. One half rehydrated with 25mL demi water, the other half freeze dried for another 24H and then rehydrated with 25mL water. After rehydration inoculated with Flavobacteria on several places.



A.8.8 / Characteristics Freeze-dried MAR medium

Alternative Medium

MAR medium in petri dish

MAR Medium (mL)	Height middle (mm)
25	3,5
20	2,8
10	1,2
FD & RH MAR Medium (mL)	**Height middle (mm)**
25	1,7
20	1,3
10	0,4
FD & RH MAR Medium FROZEN (mL)	**Height middle (mm)**
25	2,6

MAR medium in petri dish

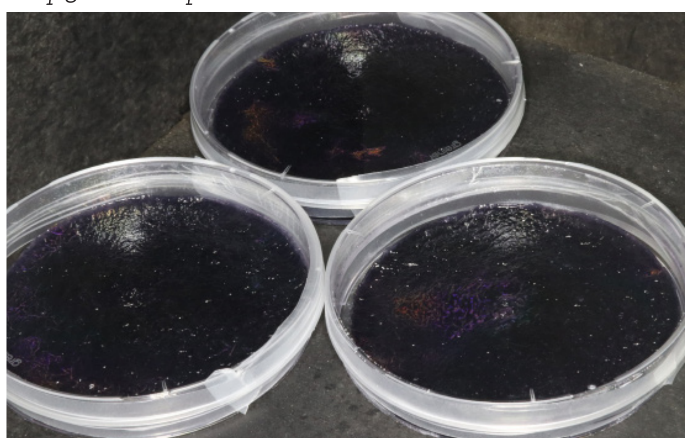
FD & RH MAR Medium (mL)	Water absorption (mL)
25	10-14
20	10
10	4
FD & RH MAR Medium FROZEN (mL)	**Water absorption (mL)**
25	15

FD = Freeze-dried
RH = Rehydrated

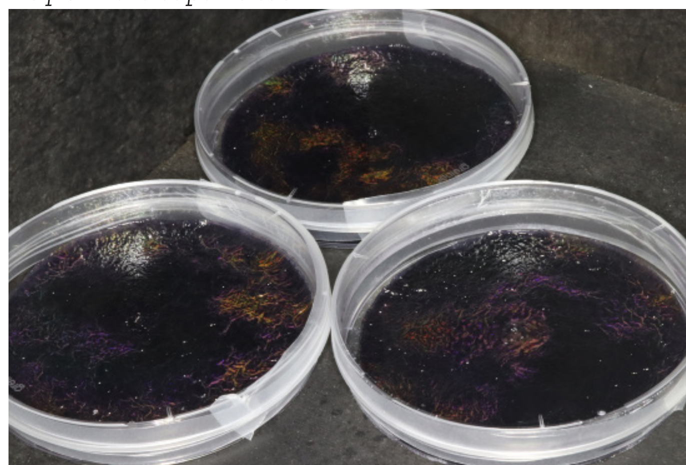
FROZEN - indicates that the sample was pre-frozen before the freeze-drying process.

A.9.1/ Rehydrated MAR + Flavobacteria (Frozen)
Integration

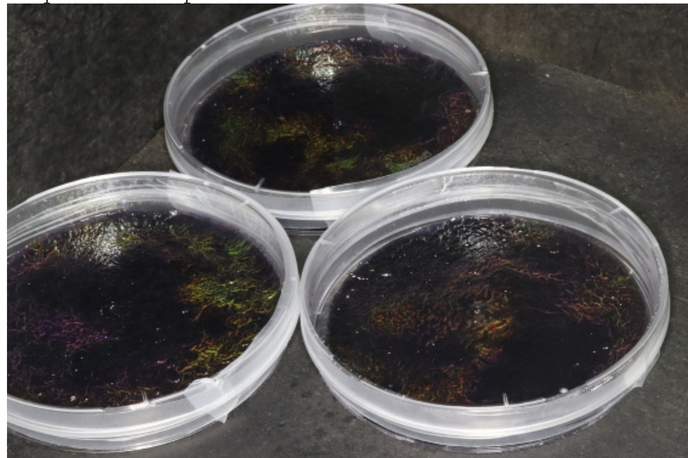
Day 3 in the capture tool



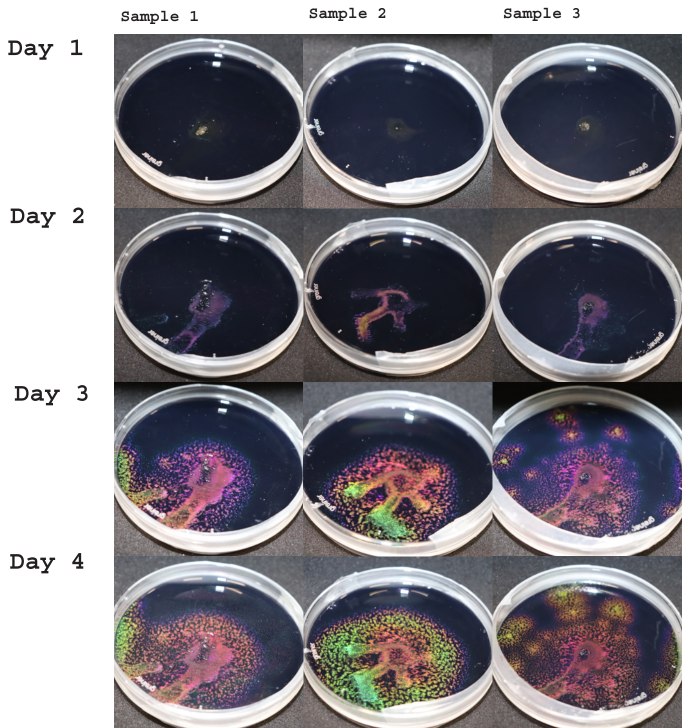
Day 6 in the capture tool



Day 8 in the capture tool

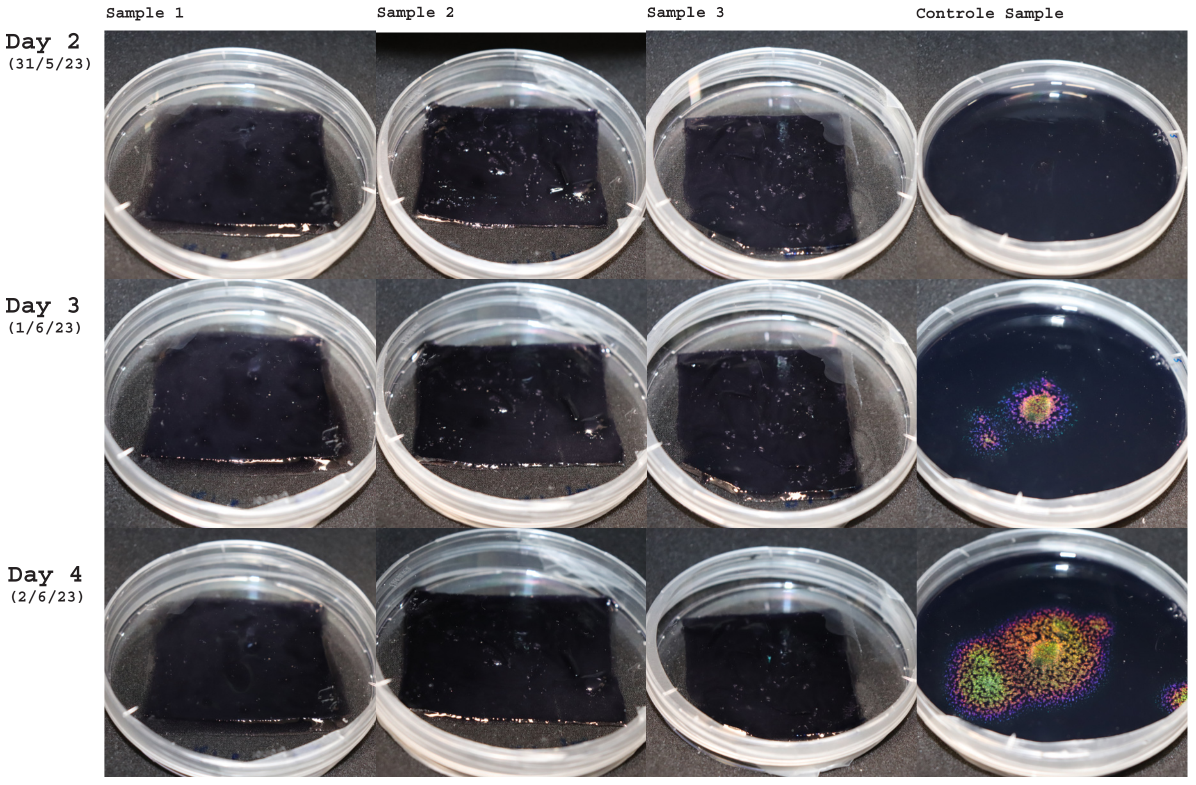


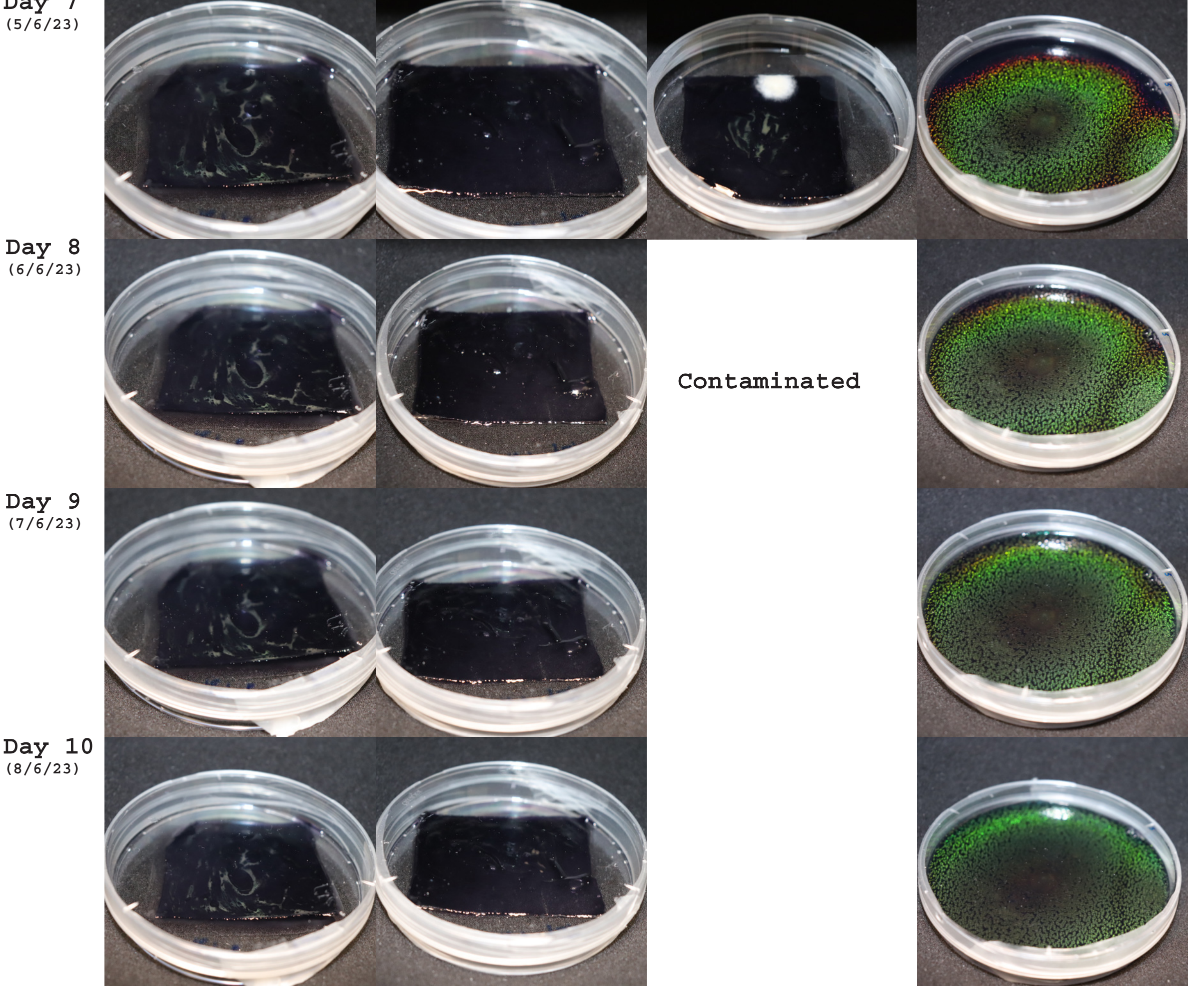
Same freeze-dried Flavobacteria on regular MAR medium (control samples):



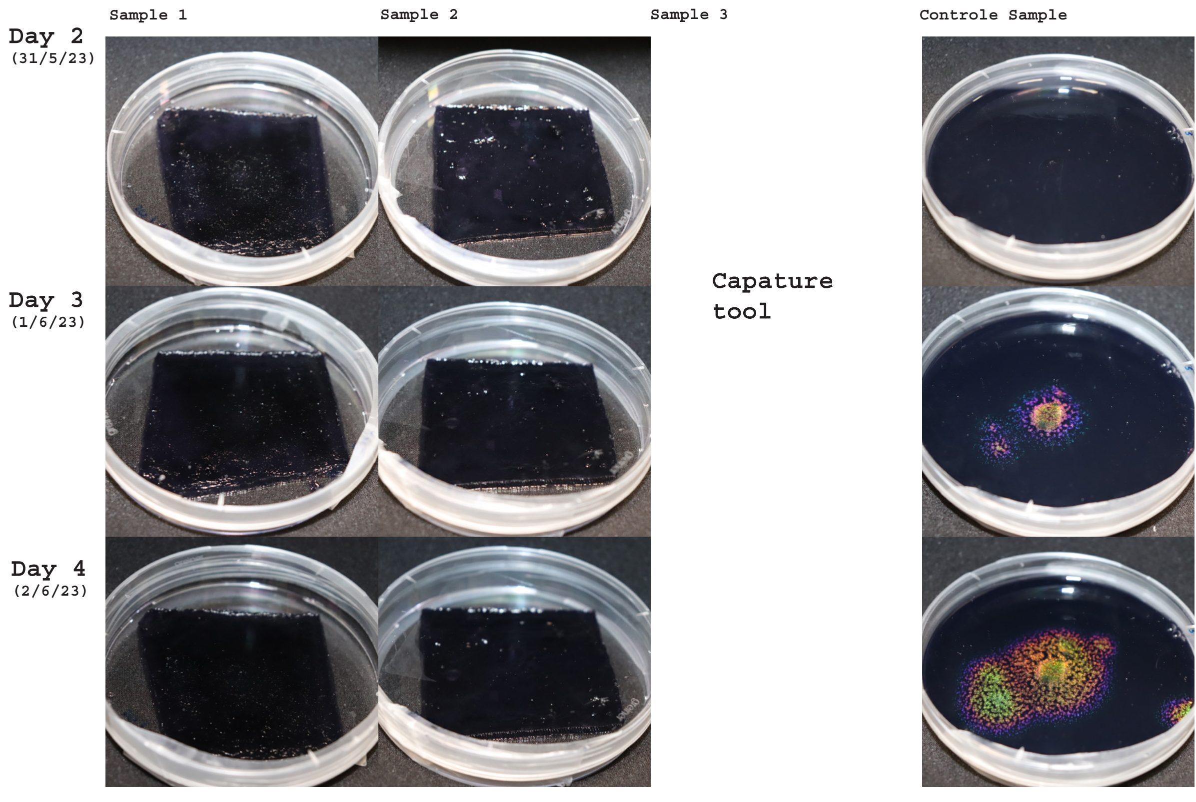
A.9.2 / Rehydrated MAR + Flavobacteria (10mL)

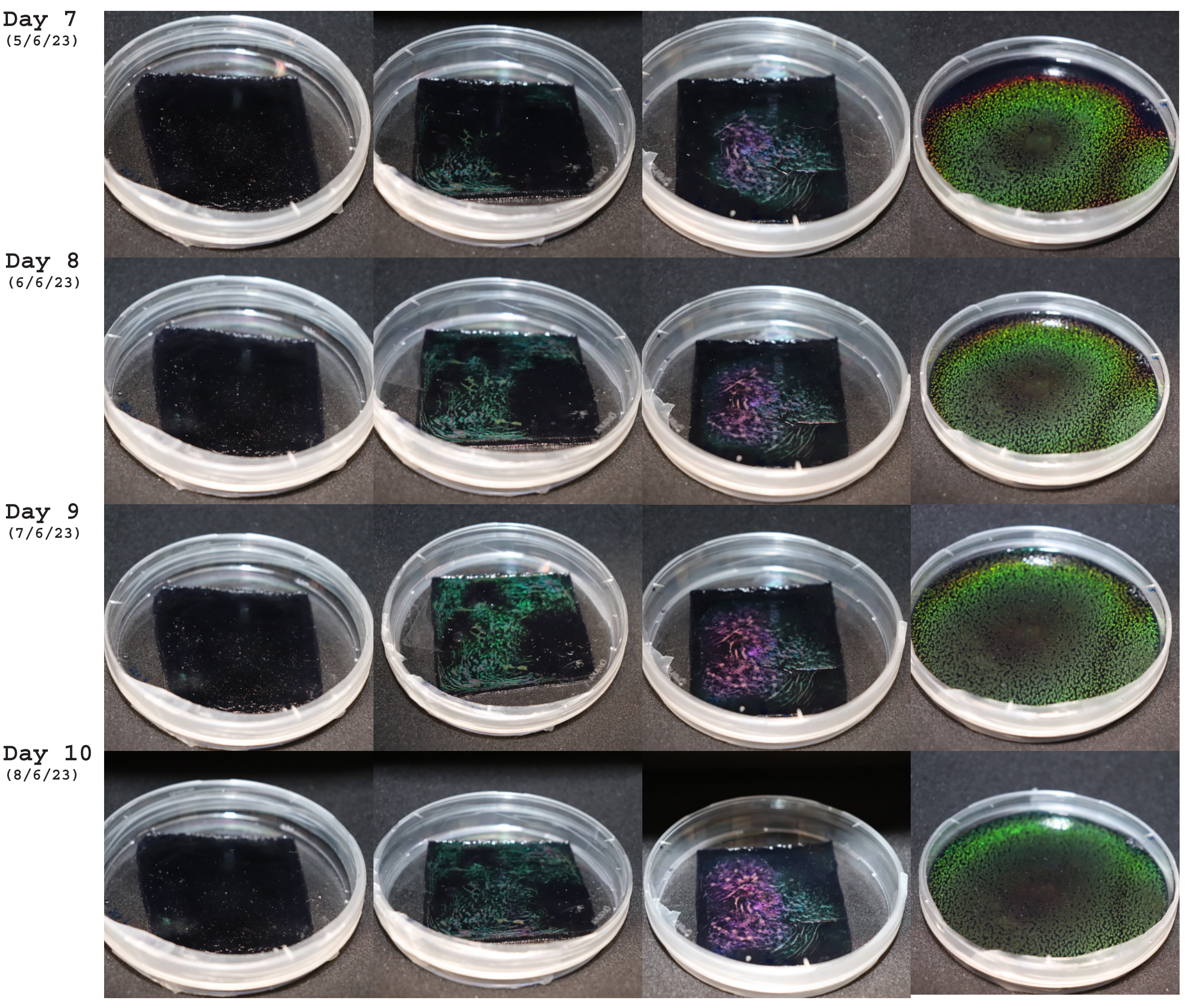
Integration





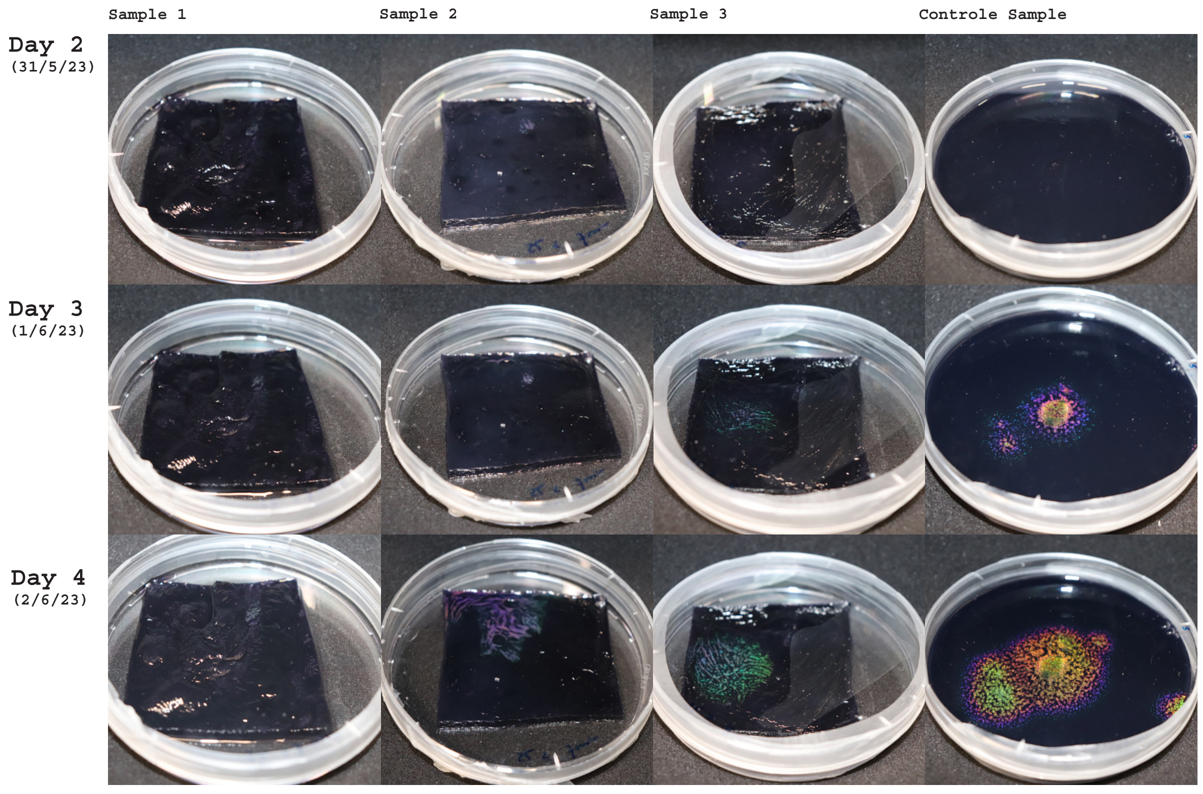
A.9.2 / Rehydrated MAR + Flavobacteria (20mL)
Integration

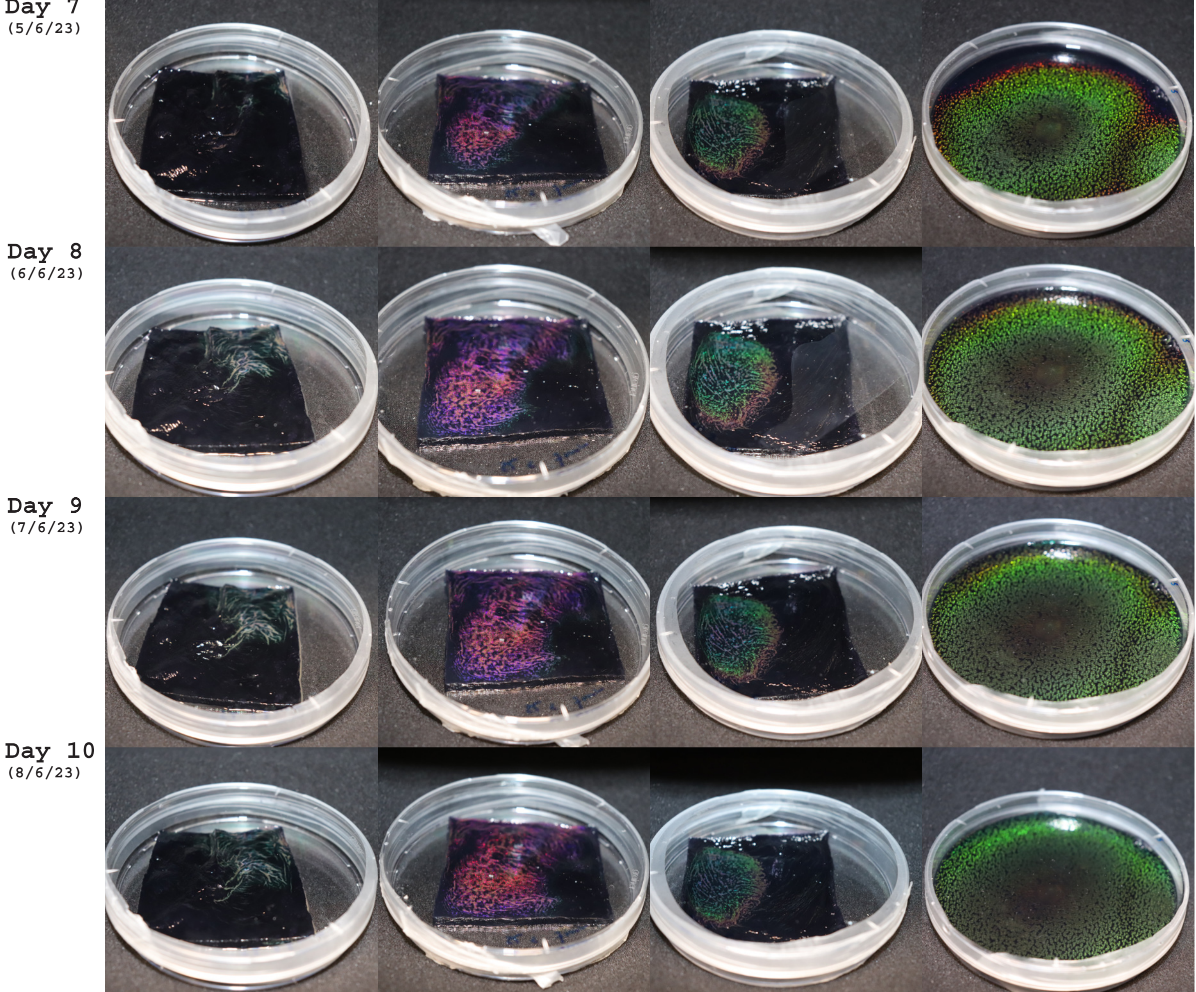




A.9.2 / Rehydrated MAR + Flavobacteria (25mL)

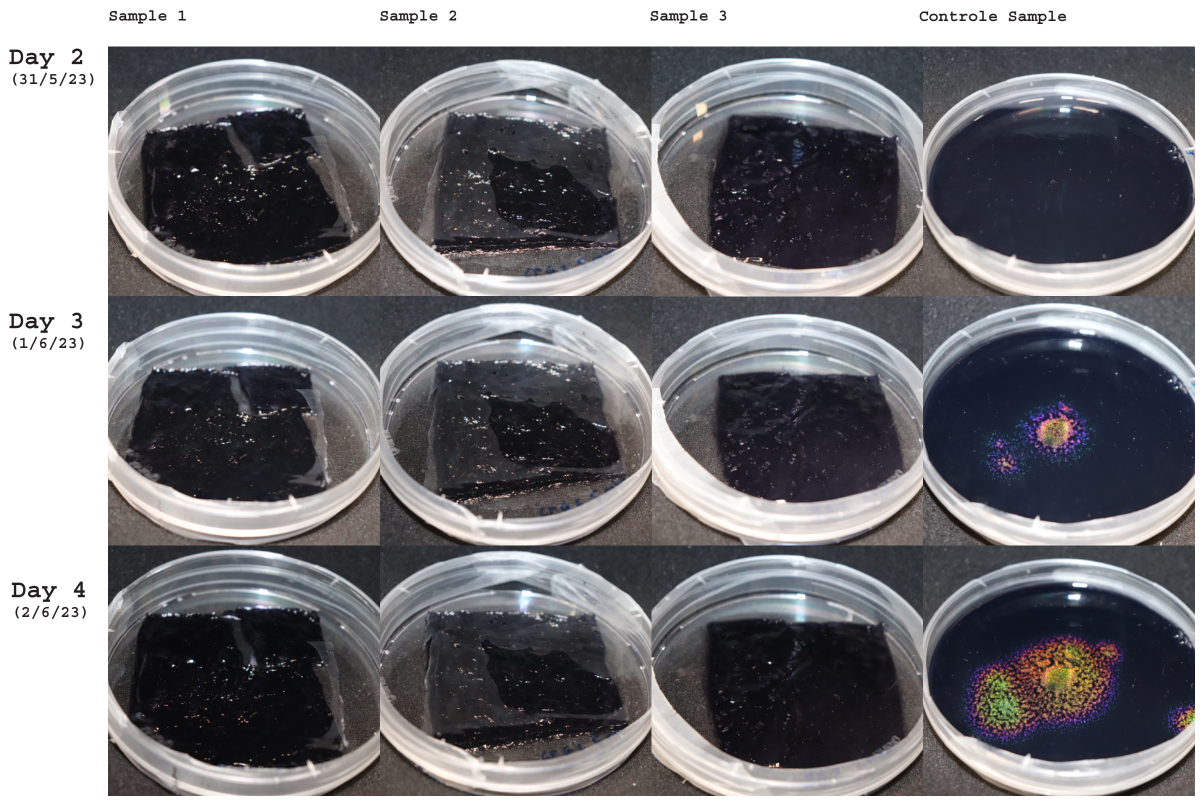
Integration

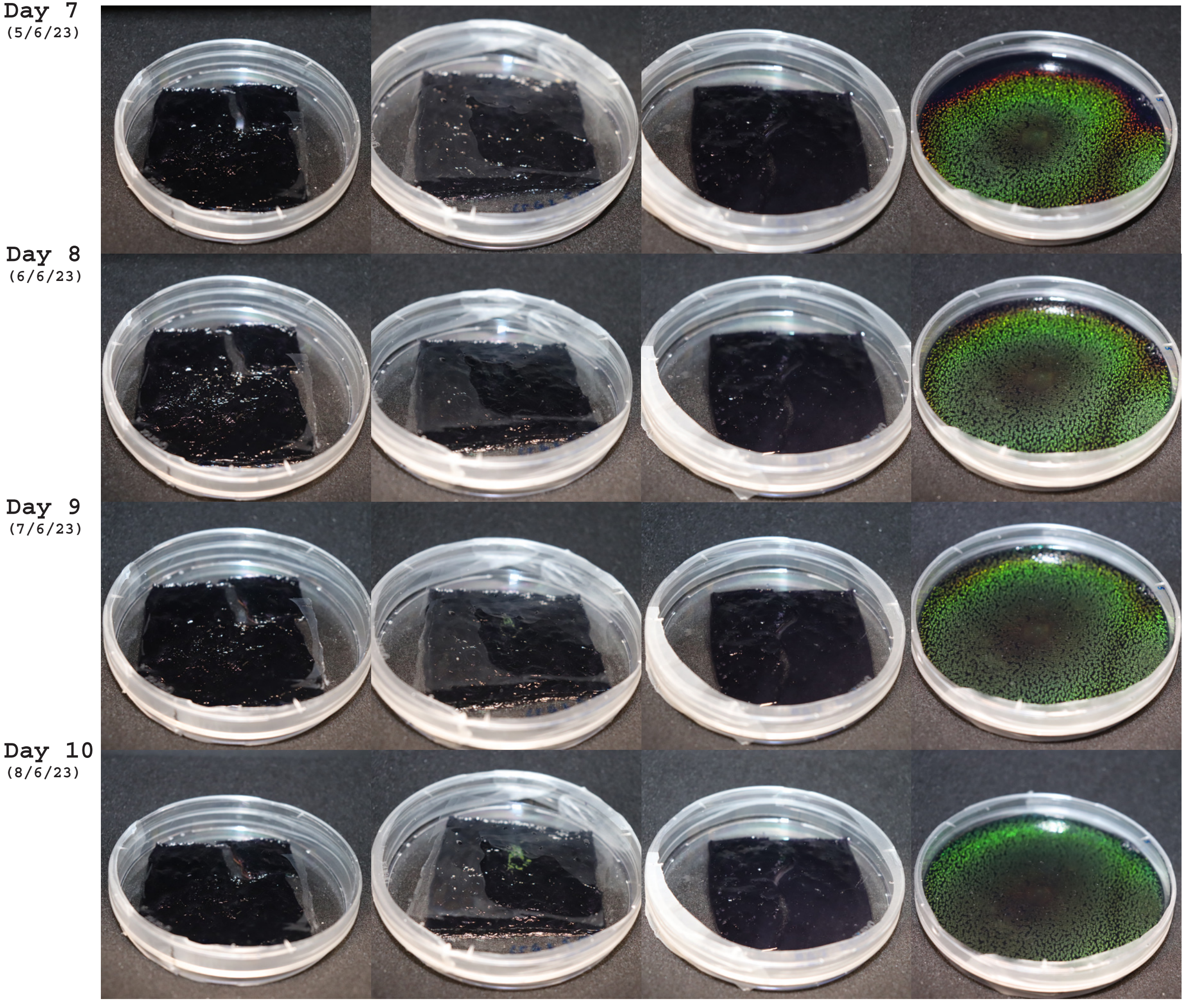




A.9.2 / Rehydrated MAR + Flavobacteria (holes)

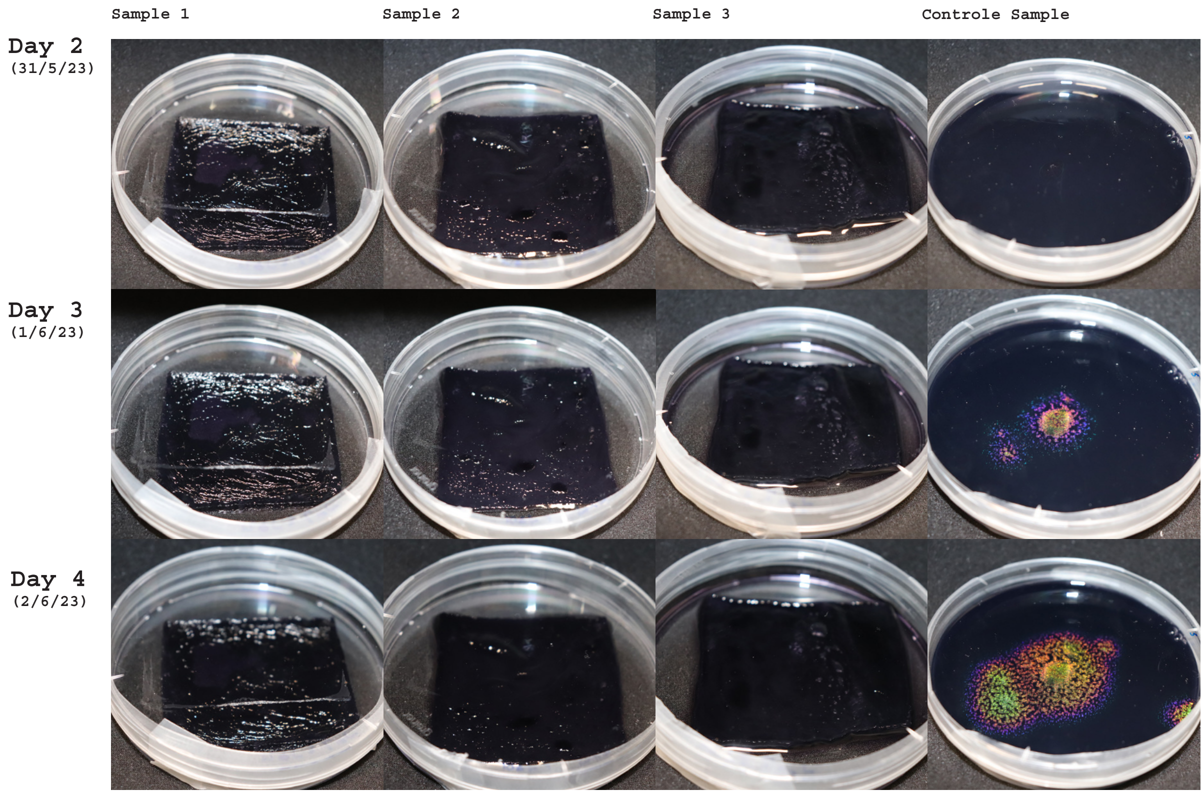
Integration

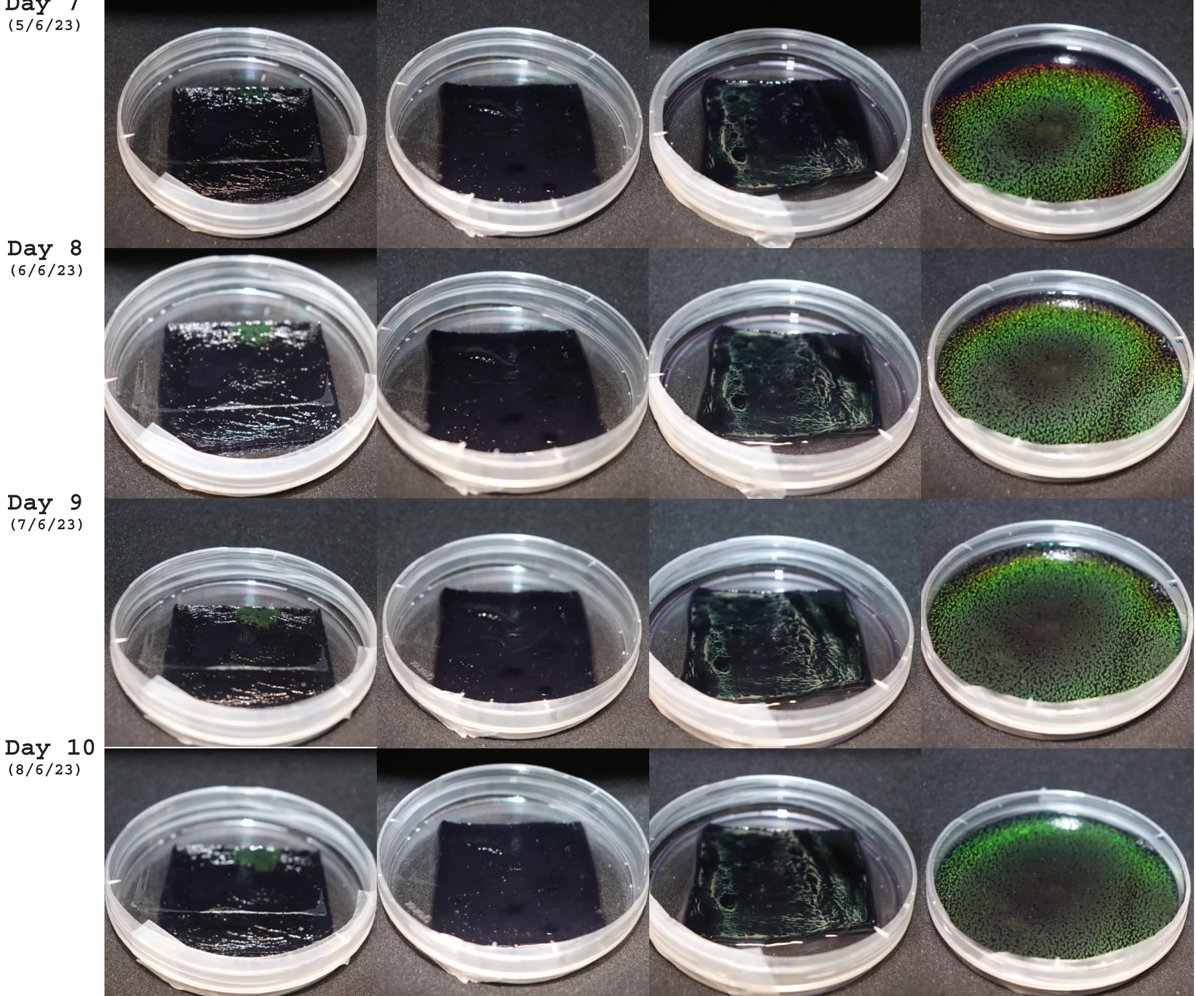




A.9.2 / Rehydrated MAR + Flavobacteria (Airflow)

Integration





To the cupboard in which all these samples have grown, farewell! :)