

Morphological Stability and Cell Density Dynamics of *Chlorella vulgaris* Bacterial Co-cultures During Laboratory Freezer Storage at 20°C with Indigenous Bacterial Consortium

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ABSTRACT

The development of microalgae-based biofuels through co-culture systems holds great potential; however, the scale-up process is often hindered by issues related to inoculum stability during storage. This study aimed to evaluate the effect of storage duration at -20 °C (for 2, 4, 6, 8, 10, and 12 weeks) on cell size and cell density of *Chlorella vulgaris* in a monoculture system compared with a co-culture system with an indigenous bacterial consortium (*Bacillus paramycoides*, *Lysinibacillus fusiformis*, and *Delftia* sp.). Optical density was measured using a UV-Vis spectrophotometer at 675 nm, while cell morphology was evaluated through microscopic observation in five fields of view. Statistical analysis using ANOVA revealed a significant decline in cell density over storage time ($p < 0.05$) throughout the 12 weeks in both systems. This structural preservation is presumed to be mediated by Extracellular Polymeric Substances (EPS) forming microalgae-bacteria flocs (MaB-Flocs) that serve as a protective physical buffer. However, viability assays conducted at week 4 showed no cell proliferation after thawing in either system, accompanied by distinct color degradation pathways. Therefore, storage at -20 °C without cryoprotectants is ineffective as a cryopreservation method and unsuitable for delaying cell density measurements, although co-cultivation effectively stabilizes cell morphology evaluations.

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INTRODUCTION

The energy transition is driving the development of biomass sources that can be produced sustainably, do not directly compete with food crops, and can be integrated with circular economy principles (Gultom, 2018). Microalgae have long been recognized as candidates for next-generation biofuel feedstocks due to their ability to convert CO₂ and light into lipid-rich biomass. *Chlorella vulgaris* is one of the most promising microalgae, with a lipid content of approximately 14%–22% (Josephine et al., 2022). Microalgae also have the potential to be cultivated on less fertile land or integrated with nutrient-rich waste. The development of microalgae-based biofuels is currently advancing and being evaluated, both in terms of opportunities and challenges. The urgency to improve productivity and production process efficiency is a primary focus for researchers (Brennan et al., 2010).

At the bioprocess level, one widely developed approach is to create a co-culture of microalgae-bacteria consortia to replicate the natural *phycosphere* and leverage mutually beneficial metabolite exchange. Co-culture is performed by growing two microorganisms simultaneously (Khaq et al., 2024). Microalgae-bacteria interactions result in a mutualistic symbiosis with key mechanisms including carbon exchange, nutrient recycling, and the utilization of metabolites as growth factors for microalgae (Ramanan et al., 2016; Yao et al., 2019; Wang et al., 2020). Co-culture can increase *C. vulgaris* biomass production compared with monoculture (Xu et al., 2024). The literature also suggests that microalgae-bacteria consortia can be engineered as *synthetic ecology* to improve productivity, culture resilience, and resource utilization efficiency. This approach aligns with the principles of the circular bioeconomy (Kong et al., 2024). Co-culture also exhibits strong biofilm functionality, increased metabolic diversity, and better resilience to environmental stressors (Tong et al., 2024). At the local scale, the discovery of indigenous vitamin B12-producing bacteria from eutrophic aquatic ecosystems is proposed as a strategy to support increased bioalgal biomass. This indicates that microbial resources can be combined to improve culture yields (Prabaningtyas et al., 2021).

Although co-culture holds great potential, the success of research and *scale-up* is often limited by more fundamental challenges, namely inoculum stability and consistency in sample handling. Technically, researchers often need to store cell cultures at low temperatures to delay subculturing, stabilize the inoculum, or wait for measurement schedules. The temperature commonly used for storage in laboratories is -20°C . Previous research indicates that cold storage at 4°C can serve as a more stable, efficient, and *repeatable* maintenance option compared to repeated subculturing (Pozzobon et al., 2024). A study on *C. vulgaris* showed that freezer storage at approximately -15°C could not guarantee viability, while storage at -80°C can lead to a decrease in viability. This implies that the selection of temperature and storage duration has the potential to alter the biological quality of the sample and *post-thawing* performance (Kapoor et al., 2019; Papadopoulos et al., 2023).

Microalgal preservation strategies focused on maintaining viability have been widely investigated. Similarly, studies on co-cultures have emphasized the impact of bacterial consortia on microalgal biomass and physiological profiles. However, research examining how storage duration at -20°C affects *co-culture* stability and how co-cultures differ from monocultures remains very limited (Kong et al., 2024). Therefore, the effects of -20°C storage on *co-cultures* need to be investigated. This study of the storage of *C. vulgaris co-cultures* with indigenous bacterial consortia aims to examine the impact of storage duration at -20°C on the stability of several parameters. The primary parameters evaluated are cell density, cell size, and cell viability. The results of this study are expected to yield data-driven recommendations regarding safe storage limits at -20°C that minimize bias in biological parameters. This study also aims to establish standard sample-handling procedures for research and applications involving the microalga (Pozzobon et al., 2024). Although microalga-bacterial *co-cultures* have been recognized for their ability to increase biomass through nutrient and growth factor exchange, and methods for microalga preservation have advanced, there is currently insufficient operational evidence regarding safe storage-duration limits at -20°C that ensure the stability of co-culture parameters.

MATERIALS AND METHODS

Experimental Design and Materials

This study on *C. vulgaris* storage was designed to examine the effect of storage duration at -20°C on cell stability. The experiment evaluated two distinct sample groups derived from monoculture and co-culture systems. In the co-culture treatment, microalgae were grown synergistically with an indigenous bacterial consortium acting as a growth-promoting agent. The consortium comprised *Bacillus paramycooides* as a nitrogen (N) fixer, *Lysinibacillus fusiformis* as a vitamin B12 producer, and *Delftia* sp. as an indole-3-acetic acid (IAA) producer.

Preparation of *C. vulgaris* Algal Culture

The *C. vulgaris* microalgae to be cultured were first rejuvenated. The rejuvenation medium consisted of 600 mL distilled water, 75 mL *C. vulgaris* culture, 75 mL Nutrient Broth (Merck), and 0.75 mL Gusrina Medium. The composition of the Gusrina medium was as follows: 39 g/L Triple Super

Phosphate (TSP); 45 g/L urea; 1 g/L FeCl₃. A total of 0.025 g/L vitamin B12 was aseptically added to the sterilized Gusrina medium (Prabaningtyas et al., 2025). *C. vulgaris* was cultured at a light intensity of 1200 lux with a 16-hour light-on and 8-hour light-off cycle, 12 hours of aeration, and a temperature of 30 °C. The *C. vulgaris* microalgal culture was harvested on the 14th day.

Preparation of Indigenous Bacterial Cultures

The consortium of indigenous bacteria used in this study included *Bacillus paramycoides*, *Lysinibacillus fusiformis*, and *Delftia* sp. Each of these bacteria was revived and purified in Nutrient Agar (Merck). Each bacterium was then cultured in Nutrient Broth (Merck) for 24 hours at 37 °C before being used in the co-culture system.

Preparation of *C. vulgaris* Co-culture with the Indigenous Bacterial Consortium

The *C. vulgaris* co-culture with the indigenous bacterial consortium was prepared by growing *C. vulgaris* together with the indigenous bacterial consortium in a single container. The co-culture composition consisted of 600 mL distilled water, 75 mL *C. vulgaris* culture, 75 mL standard bacterial culture (0.5 McFarland), and 0.75 mL Gusrina medium. The *C. vulgaris* culture used for the co-culture was 7 days old. The *Bacillus paramycoides* and *Lysinibacillus fusiformis* bacteria used were 1 day old (24 hours), while the *Delftia* sp. was 3 days old.

Sampling and Measurement

Culture samples were collected simultaneously, aliquoted into separate tubes, and stored at -20°C. Samples were incubated under frozen conditions for 2, 4, 6, 8, 10, and 12 weeks to evaluate cellular stability. The primary parameters evaluated were cell density and cell size. Cell density was quantitatively analyzed to monitor population consistency following the storage period. Absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 675 nm, and values were subsequently converted into cell counts based on a calibration curve generated using a hemocytometer at the beginning of the study. Cell size was determined through microscopic measurement in five distinct fields of view per sample to identify potential morphological changes induced by cryopreservation. Both parameters were comprehensively evaluated for monoculture and co-culture treatments to ensure that storage at -20 °C maintains stable cellular parameters and minimizes biological bias.

Data Analysis

Cell density and cell size measurements were analyzed using one-way ANOVA, following prerequisite tests for normality and homogeneity of variance. Data were analyzed using a one-way ANOVA at a 95% significance level ($\alpha = 0.05$), followed by Welch's and Tukey's post hoc tests.

RESULTS AND DISCUSSION

Cell Size

Analysis of the *C. vulgaris* cell size graph revealed significant differences in both the monoculture and co-culture systems. *C. vulgaris* cells in the co-culture system exhibited a larger diameter, remaining stable within a range of 5.4 to 6.8 µm, whereas in the monoculture system, cell sizes ranged from 2.9 to 3.7 µm. The ANOVA results, followed by Welch's post-hoc test, indicated a significant change in size ($p < 0.05$) for both treatment groups during the storage period. The graph of average cell sizes during storage is presented in Figure 1. Visualizations of *C. vulgaris* cells in the co-culture and monoculture systems throughout the storage period are shown in Figure 2.

Statistical analysis revealed that storage duration at -20 °C significantly affected the cell size of *Chlorella vulgaris* ($p < 0.05$) in both the monoculture and co-culture systems. This time-dependent variation can be attributed to slow freezing dynamics and ice recrystallization phenomena during long-term storage. Because storage at -20 °C remains above the glass transition temperature of the microalgal cytoplasmic matrix, an unfrozen water fraction persists and remains active at the microscale. As the storage period extended to 12 weeks, the gradual growth and recrystallization of

extracellular ice crystals progressively induced hypertonic osmotic pressure, drawing water out of the cells via osmotic dehydration while exerting direct mechanical stress on the cell wall (Day et al., 2021). Consequently, volume fluctuations and structural cell compression occurred progressively over time in both treatment groups.

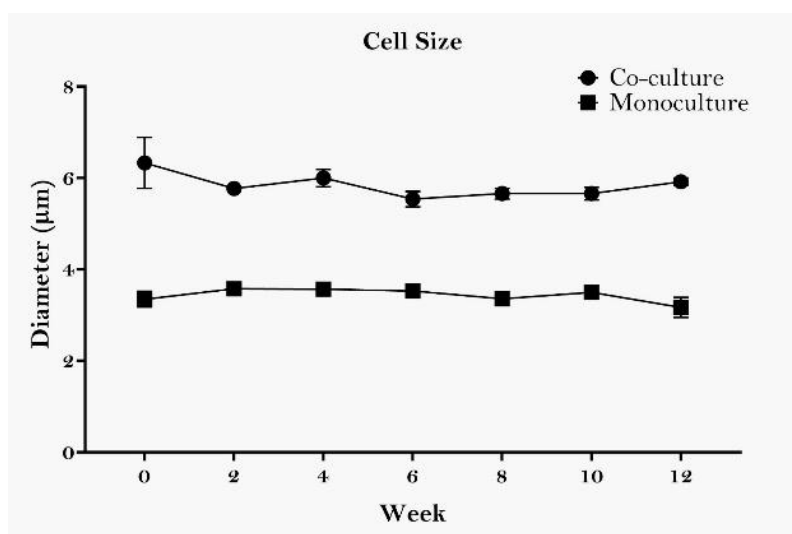


Figure 1. Graph of Cell Measurement Results

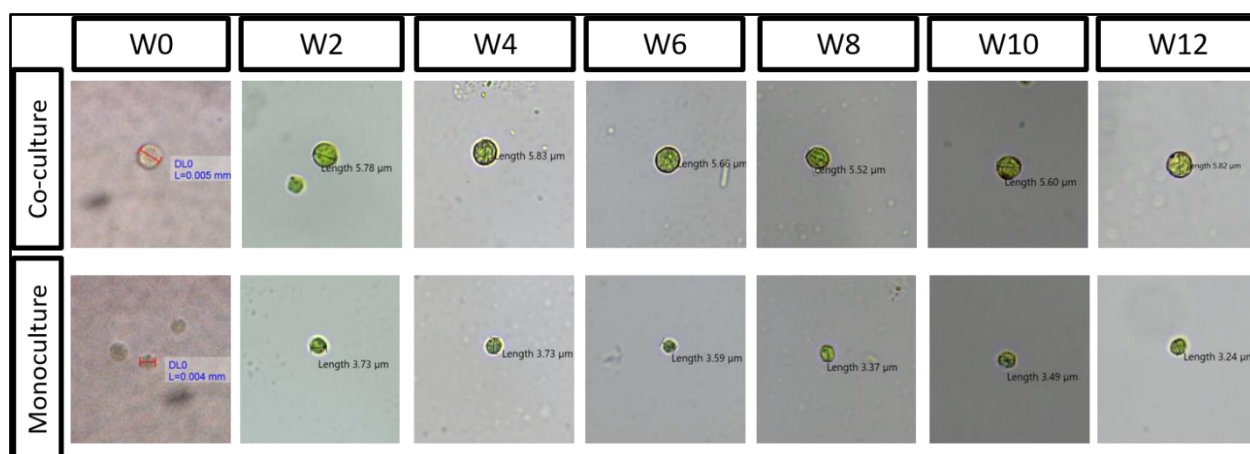


Figure 2. Visualization of *Chlorella vulgaris* during the storage period

Despite these time-dependent size changes across both systems, a distinct difference in morphological resilience was observed between the two culture conditions. *C. vulgaris* cells in the co-culture system exhibited larger overall diameters, remaining within the range of 5.4–6.8 µm, with microscopics observation showing intact, rounded cell shapes similar to non-frozen control cells. Conversely, cells in the monoculture system were markedly smaller, ranging from 2.9 to 3.7 µm, and frequently displayed structural shrinkage and loss of spherical shape post-thaw. Although *C. vulgaris* possesses a rigid microalgal cell wall composed of thick cellulose microfibrils, which helps preserve its three-dimensional structure even after internal cell lysis (Pozzobon et al., 2024), direct exposure to freezing stress in the absence of protective bacterial associations triggers severe cellular dehydration and damages the cell wall microfibrils, resulting in pronounced cell deformation (Kapoor et al., 2019; Pozzobon et al., 2024).

The morphological stability observed in co-cultures may be driven by microenvironmental interactions within the algal–bacterial system (Ramanan et al., 2016). In co-culture systems, interactions with indigenous bacteria have been reported to trigger the secretion of Extracellular Polymeric Substances (EPS), which envelop microalgal cells within a protective biofilm or floc matrix (Flemming et al., 2007). Although EPS production was not directly quantified in the present study,

we hypothesize that EPS secretion by the bacterial consortium contributes to the observed structural preservation based on findings reported in similar systems (Ramanan et al., 2016; Flemming et al., 2007). This EPS matrix could potentially act as both a physical buffer and a natural cryoprotectant, mitigating osmotic stress and shielding cell walls from mechanical damage caused by ice crystal growth during storage at -20 °C. Conversely, in monoculture systems lacking bacterial association, cells are directly exposed to freezing stress. This exposure triggers cellular dehydration and damages cell wall microfibrils, leading to cell shrinkage post-thaw (Kapoor et al., 2019; Pozzobon et al., 2024). To validate this hypothesized mechanism, direct quantification of EPS, such as through a phenol-sulfuric acid assay, is recommended for future studies.

This hypothesized matrix-mediated protection is further supported by the distinct sedimentation behavior observed during storage. In the co-culture system, distinct green biomass precipitation observed at the bottom of the microtubes, whereas the monoculture system remained evenly distributed and homogeneous (Figure 3). Biomass precipitation in co-culture indicates physicochemical interactions between *C. vulgaris* and the indigenous bacterial consortium. Bacteria such as *Lysinibacillus fusiformis* possess a high capacity to secrete EPS, which alters environmental viscosity and intercellular interaction dynamics (Mahendran et al., 2013), thereby facilitating adhesion between bacterial membranes and cellulose in the algal cell wall (Flemming et al., 2007).

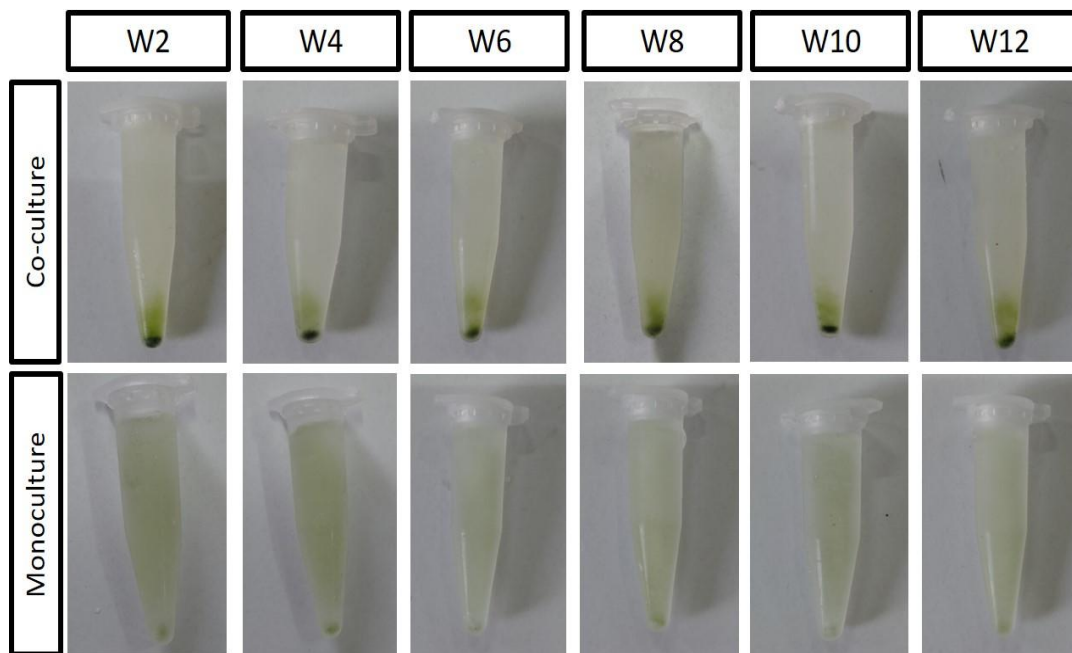


Figure 3. Visualization of *Chlorella vulgaris* sedimentation during the storage period

These EPS-mediated interactions trigger the formation of macroaggregates known as Microalgae-Bacteria Flocs (MaB-Flocs), representing a microbial survival strategy in response to environmental stress prior to freezing (Wieczorek et al., 2015). The resulting MaB-Flocs possess a higher specific gravity and mass density than individual cells, exceeding single-cell buoyancy and causing gravitational sedimentation to the bottom of the tube (Kong et al., 2024). In contrast, monoculture systems lack EPS-producing bacteria. Although *C. vulgaris* exhibits natural autoflocculation in response to environmental stressors such as fluctuations in temperature, pH, and ionic strength (Ahmad et al., 2022; Spain et al., 2024), this intrinsic autoflocculation yields substantially smaller aggregates than bacterial co-flocculation, leaving the monoculture suspension visually homogeneous (Figure 3).

Cell Density

Based on the statistical analysis, the population density of *C. vulgaris* cells decreased in both systems over 12 weeks of storage at -20 °C. In the monoculture system, ANOVA followed by Welch's

post-hoc test demonstrated a significant effect of storage duration on cell density. Similarly, in the co-culture system, ANOVA followed by Tukey's *post-hoc* test revealed a significant decline in cell density. The mean cell density of *C. vulgaris* during storage is presented in Figure 4 for the monoculture system and Figure 5 for the co-culture system.

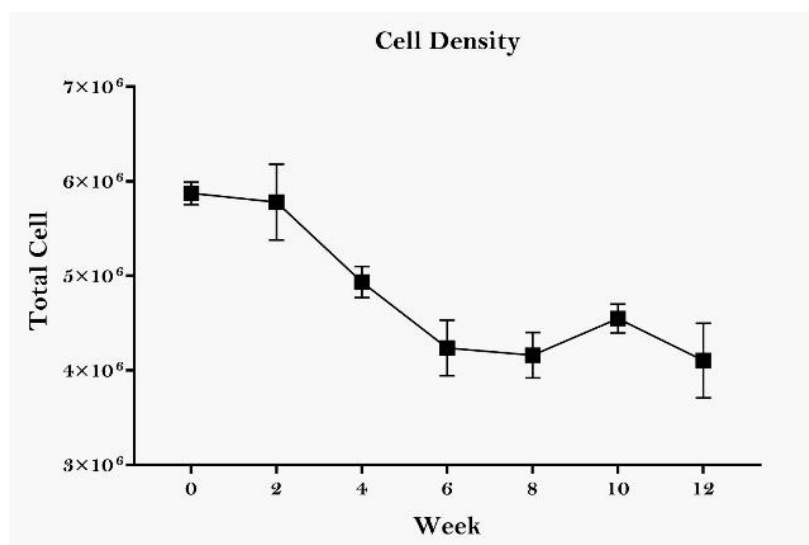


Figure 4. Cell Density Graph for The Monoculture System

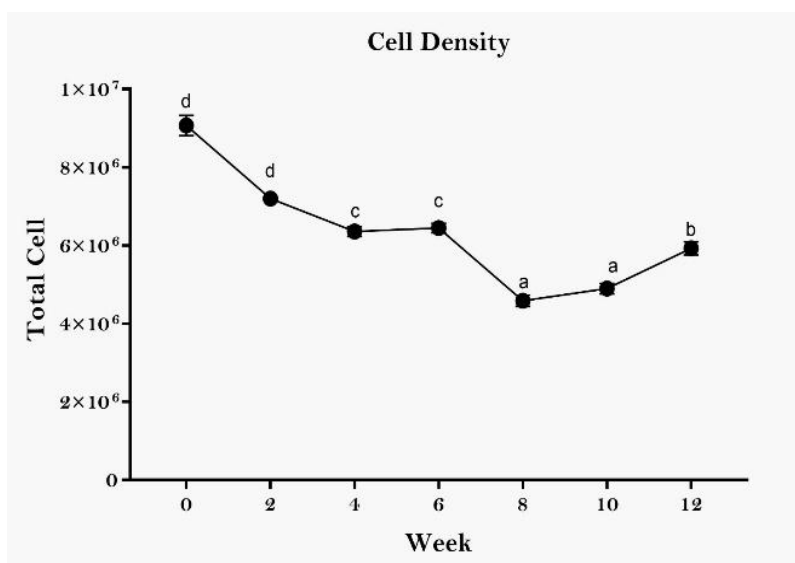


Figure 5. Cell Density Graph for The Co-culture System

Data analysis indicates a decrease in viable *C. vulgaris* cell density as storage time progressed in both systems. This reduction demonstrates that storage at $-20\text{ }^{\circ}\text{C}$ without the addition of cryoprotectants is incapable of maintaining long-term cell viability. The decline in cell density is associated with physical damage at the cellular level caused by water freezing. Storage at temperatures between $-15\text{ }^{\circ}\text{C}$ and $-20\text{ }^{\circ}\text{C}$ triggers the formation of intracellular and extracellular ice crystals, compromising the integrity of the algal plasma membrane. These ice crystals induce severe cellular dehydration, perforate the protective membrane, and cause leakage of genetic material and cytoplasm (Kapoor et al., 2019).

Cell lysis in *Chlorella vulgaris* can ultimately lead to microalgal cell death, a phenomenon supported by observations from the initial viability assay conducted after 4 weeks of storage. Originally, viability testing was designed to be performed at monthly intervals throughout the storage duration. However, the first assay at Week 4, utilizing a 7-day incubation period in a mixture consisting of 800 μL of distilled water, 200 μL of culture sample, and 1 mL of Gusrina medium yielded

no indication of microalgal cell proliferation, as evidenced by the lack of an increase in green color intensity. Consequently, the quantitative viability assay was discontinued for subsequent time points and excluded as a primary parameter. Nevertheless, qualitative descriptive observations at the end of the incubation period revealed distinct color variations between treatment groups (a yellowish hue in the co-culture versus pale green in the monoculture; Figure 5). These observations were therefore retained as supporting data to illustrate post-thaw cellular responses. Although qualitative, these color variations provide valuable preliminary insights into post-storage cell breakdown dynamics. For future studies, incorporating quantitative measurements such as spectrophotometric quantification of chlorophyll *a/b* ratios or oxidative stress markers is strongly recommended to objectively confirm these specific cell degradation pathways.

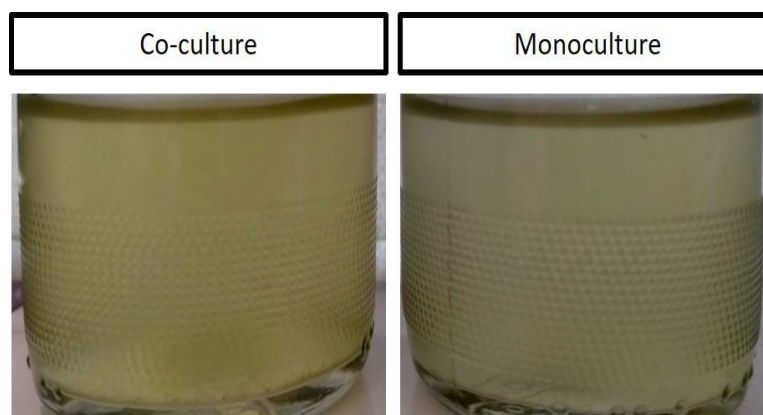


Figure 5. Differences in color from the viability test

The failure of this viability assay demonstrates that morphological stability does not necessarily reflect cellular health status. Storage at -20 °C results in uncontrolled freezing rates, causing irreversible damage to chloroplasts and mitochondria. Fatal damage to the photosystem renders the microalgae incapable of resuming photosynthesis post-thaw (Gwo et al., 2023). This scientifically confirms that *C. vulgaris* cells appearing perfectly round under a microscope are not necessarily viable. Furthermore, a methodological limitation of the present study lies in the reliance on UV-Vis spectrophotometry for cell density estimation. Spectrophotometric measurements quantify overall cell density based on culture turbidity, reflecting total cell biomass rather than metabolic viability, unlike vital staining techniques such as Trypan Blue exclusion, optical absorbance cannot differentiate between living and dead cells. Consequently, the observed decline in cell density reflects a reduction in intact cell population and directly correlates with cell death in *C. vulgaris*.

The color response of the cultures upon transfer to fresh medium indicates divergent decomposition pathways. The yellow color in the microalgal culture indicates oxidative stress and structural damage to thylakoids within chloroplasts (Elisabeth et al., 2021). Chlorophyll is responsible for photosynthesis, and microalgal cells are also surrounded by pigment-producing structures (Tan et al., 2017). The yellowing observed in the co-culture system was also significantly influenced by the survival of remaining bacteria. When microalgal cytoplasm ruptures post-thawing, internal organic matter leaks into the medium. Bacteria with higher freeze tolerance utilize these nutrients as substrates for decomposition. The simultaneous decomposition of algal organic matter and degradation of secondary pigments transform the sediment into a cloudy yellowish hue.

In the monoculture treatment, the culture exhibited a pale green color upon regrowth attempt. This pale green color serves as a biological indicator of pure chlorosis (Prabaningtyas et al., 2021). Cold-induced injury causes chlorophyll to lose its central magnesium core. The chlorophyll pigment degrades slowly in the absence of bacteria to consume the cellular debris, resulting in a pale culture (Pozzobon et al., 2024). The pale color in monocultures also suggests a lack of essential nutrient sources. Chlorophyll synthesis and healthy microalgal metabolism rely heavily on external supplies of nutrients, such as Vitamin B12 (Papadopoulos et al., 2023; Prabaningtyas et al., 2025), which is

synthesized by bacteria and archaea in nature (Watanabe et al., 2018). Freezing conditions combined with the absence of consortium-derived nutrients cause the algal cells in the monoculture to turn pale.

CONCLUSION

Storage at -20 °C without cryoprotectants is ineffective both as a cryopreservation method and as means of delaying population density measurements of *Chlorella vulgaris*. Over 12 weeks of storage, a significant decline in cell density occurred ($p < 0.05$), accompanied by a complete loss of viability in both monoculture and co-culture systems due to osmotic dehydration and membrane lysis. Nevertheless, the co-culture system with an indigenous bacterial consortium maintained better morphological stability than the monoculture, owing to the physical protection provided by the EPS matrix (MaB-Flocs). Therefore, storage at -20 °C is only recommended for delaying short-term observations of cell morphology and size in co-culture systems. For future research, the use of vital staining methods such as Trypan Blue is strongly recommended to further investigate cell viability.

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DECLARATIONS

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- Conflict of Interest** : The authors declare no conflict of interest.
- Data Availability** : The raw morphological data are available from the corresponding author upon reasonable request.
- Ethical Approval** : No ethical approval for this research
- Declaration of AI Use** : AI tools (Gemini and DeepL) were used for spelling checks and grammar corrections. The authors reviewed and take full responsibility for the final content.

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