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Post Cryopreservation Growth Kinetic and Photosynthetic Assessment of an Acid Tolerant Strain of *Stichococcus bacillaris*

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Abstract

Preserving microbial diversity has become a strategic undertaking. Thus, *ex situ* microalgal culture conservation results in strategic and functional resource in both biodiversity protection and application domains. Cryopreservation of microalgae has been practiced since the 1960s and is now considered the optimal preservation strategy. Furthermore, the overall monitoring during growth of cultures after freezing/thawing protocols was hardly investigated and there is poor evaluation related to preserve especially the photosystem apparatus. The present study focuses on *Stichococcus bacillaris* as case study for short-term cryopreservation at −80 °C storage. Various freezing pretreatments using cryoprotective agents, and two thawing methods were compared introducing a novel variable to evaluate viability recovery and assessing growth kinetics of cultures immediately after thawing and after a series batch cultivation. Photosynthetic rate and pigments assessment were proposed to evaluate hidden metabolic cell damage. Results underline cryoprotective agents can increase the kinetic recovery of preserved cells in terms of reduction of lag phase during batch cultivation tests: the use of dimethyl sulfoxide and glycerol granted a growth comparable to unpreserved cells when sudden thawing occurs after 24 hours of storage, but recovery after preservation is less sensitive to cryoprotective agents when gradual thawing and 1 month of storage is considered. However, cells are always able to restore their physiological path-

ways even without agents, so their kinetic effect has been proved and quantified. Interestingly, both the photosynthetic efficiency and the ratio between total chlorophyll and carotenoids are comparable (0.75 F_v/F_m, 2.2 ± 0.25 g/g) to unpreserved cells and they are unsensitive to chosen agents, but the ratio between chlorophyll *a* and chlorophyll *b* was clearly altered (up to 10 times), suggesting that photoactive pigments relative proportions can result in similar growth kinetic performances. Long-term studies will be carried out to assess whether the differences found could cause chronic damage to photosystem efficiency of *S. bacillaris* cultures.

Keywords

Microalgae, *Stichococcus bacillaris*, Cryopreservation, Growth Kinetic, Photosynthetic Rate

1. Introduction

The term microalgae is traditionally used to describe photosynthetic eukaryotic microorganisms, including cyanobacteria [1] [2]. These microorganisms represent a significant biodiversity resource in terms of species number, morphology, biochemical pathways, but also in terms of ecological roles and applications [3]. All microalgae are autotrophs that photosynthesize through their pigments using them as light-harvesting molecules. Chlorophylls, carotenoids, and phycobilins are the three main classes of pigments that can be present in the photosynthetic apparatus. Chlorophyll *a* is present in all microalgae as a part of the reaction center pigment-protein complexes, and in light-harvesting antennae it is accompanied by chlorophyll *b* or chlorophyll *c*, especially in green algae. As photoprotectors, carotenoids are essential for protecting against excess irradiance, chlorophyll triplets, and reactive oxygen species. Phycobilins are the main antenna pigments of red algae and cyanobacteria. It is worth noting photosynthetic efficiency depends on all above-mentioned molecules [4], and is responsible for nearly half the world's primary production [5].

In addition to pigments, other biomolecules highlight the significance microorganisms and their various applications, which are also of interest for commercial purposes. In the agricultural sector, microalgae produce bioactive substances such as phytohormones that support plant growth [6]. Microalgae are also utilized as a human food source for their high nutritional value due to their protein and vitamin content [7] [8], and can be used in a wide range of medical applications as useful sources of compounds with antioxidant, anticancer and anti-inflammatory properties [9]. Due to their lipid content and their adaptability to different environmental conditions, some microalgae can also provide a new source of biomass for fuel production with significant advantages [10] [11] [12] [13] [14]. For instance, the green unicellular microalga *Stichococcus bacillaris* is a potential candidate for commercial scale cultivations also devoted to biodiesel

production and several biotechnological applications [15] [16]. Clearly, stability and preservation of microalgae become relevant to exploit their biotechnological potential.

The preservation of the ecosystem and ecosystem services is the main priority in life science [17]. However, maintaining microbial resource *in situ* is extremely challenging. Thus, *ex situ* microalgal culture conservation results in strategic and functional resource in both biodiversity protection and application areas. In terms of biodiversity, conservation allows to preserve the genetic materials and the morphological functional characteristics of microalgae against contamination and environmental and ecological change that could cause the extinction of previously isolated organisms over time [17]. The World Federation for Culture Collections (https://wfcc.info/) is actively involved in supporting the establishment of culture collection and relative services. Strain conservation is also the first step for any kind of biotechnological exploitation [18]. However, *ex situ* maintenance is not necessarily successful. Preservation methodologies can create a stressful condition within the cells inducing a state of dormancy, or viable but not culturable, in some fraction of preserved cells according to Hoefman *et al.* [19]. Additionally, excessive costs for the required equipment remain one major problem and application limit.

To date, several methods are available for the maintenance of microalgal stock cultures: routine serial subculture, freeze-drying or lyophilization, and cryogenic storage [20]. Routine serial subculture is the earliest maintenance method. It is achieved through the continuous transfer of culture and allows the maintenance of actively growing cultures. However, this method requires routine manipulation of cultures by workers, which increases human error, contamination, and genetic drift [21]. Freeze-drying or lyophilization was therefore introduced. This method is commonly used to preserve bacterial cultures and has been successful in preserving certain strains of cyanobacteria, but there are numerous eukaryotic microalgae that cannot survive to lyophilization [22].

Cryopreservation is now considered by most practitioners at the Biological Resource Centre (BRC) (https://www.oecd.org/) to be the optimal preservation strategy for microorganisms, which can vary from the use of a domestic freezer to ultra-low temperature storage below −196˚C using liquid nitrogen. The use of ultra-low temperature allows the long-term maintenance of cultures in a state of "suspended animation" with no significant reduction in viability after storage [23], but the genetic profile after prolonged cryopreservation has not been yet investigated [20]. Cryopreservation of microalgae was performed since the 1960s providing several advantageous aspects in term of convenience for long-term stability [24], but disadvantages cannot be underestimated. This method requires dedicated equipment and continuous liquid nitrogen supply for sample storage, resulting expensive particularly at the laboratory scale, but also for large scale production by companies. The liquid nitrogen market will increase up to a CAGR around 5.28% in 7 years up to 2029, and it is expected to reach 25.65 USD

billion by 2029 (https://exactitudeconsultancy.com/it/). Additionally, the manipulation of liquid nitrogen poses potential health hazards. Moreover, several algal labs, which are also involved in biotechnology applications, are seeking strategies suitable for short-term conservation. They choose higher temperature of preservation ranging from −15˚C to −80˚C [23]. It should be noted that domestic freezers (−15˚C to −20˚C) can only be used for 24 hours of storage, after which there is a significant decrease in cell viability. In the study reported by [23], loss of viability after 1 and 4 months of storage at −15˚C was demonstrated using an agar plate assay. This suggests that this approach is fundamentally unreliable. Several possibilities of microalgal culture storage at −80˚C freezer have been reported [23] [25] [26].

The achievement of cryopreservation depends on microalgal species, cell concentrations, and the eventual addition of exogenous cryoprotective agents (CPAs). The study [27] showed that the highest recovery after preservation was obtained at a cell concentration below 10×10^6 cells·mL^{-1}. Indeed, the cell viability lowered from ~37% to ~24% when cell concentration stored increases from 1×10^6 to 10×10^6 cells·mL^{-1}. Cryopreservation promotes ice crystal formation in the preserved cells subject to cryogenic temperatures causing cell damages [17]. Several reviews on cryoprotective agents have reported that the use of cell-penetrating agents can lead to increased cell viability. However, it should be noted that the addition of cryoprotective agents in cell cultures may also result in damage over time. Kugler *et al.* [25] showed that the vegetative cells of *Lobosphaera incisa* exhibited tolerance to extreme low temperature without cryoprotective agents' addition, Bui *et al.* [27] reported very low viabilities of some green algae after preservation without pretreatment with CPAs compared to others with CPAs, proving these can help cells growth recovery. Ice crystal formation can be avoided using dimethyl sulfoxide (DMSO) and methanol, while sugars' addition can lower the water freezing point [27]. Successful cryopreservation also depends on the methods of thawing to minimize photooxidative damage [28]. After storage at −80˚C in a freezer, both transferring cultures to +4˚C reported by [25], and immersing cultures into a 35˚C water bath, as reported by [26], are efficient methods for preserving cultures. However, it is important to note that there is currently no standardized operational protocol for cryopreservation of cultures. In addition, beside the method of preserving, many other aspects may influence the success of preservation [19], representing an empirical research field. Comparing or applying data from literature results very hard. For instance, the cells adaptation to fresh medium after thawing, the certainty of cells growth stability, and photosynthetic apparatus efficiency have not been completely proven in every case study [17] [26].

The present work aims to introduce the attempt to cryopreserve a strain of *Stichococcus bacillaris* Nägeli belonging to The Algal Collection at the University of Naples Federico II (ACUF) [29]. ACUF is an acknowledged thermoacidophilic microalgae collection resulting from the exploration of several ex-

treme sites which led to isolate strains belonging to *Chlorophyta*, *Rhodophyta* and *Bacillariophyceae* [30] [31]. *Stichococcus* is a genus of green algae that is widespread across several continents and tolerant of changes in temperature, salinity, and pH [32]. In ACUF are presently maintained 16 strains of *S. bacillaris*, also isolated from italian low pH environments (pH < 3.0), that represent a unique pool of biodiversity deserving tailored strategies of preservation.

Strains of *Stichococcus bacillaris* have also been isolated from Antarctica, showing a good adaption to extremely low temperatures and making it a potential candidate for cryopreservation [33]. It is worth noting that Holm-Hansen [34] reported a historical attempt to preserve *S. bacillaris* at ultra-low temperature below −196˚C, dating back to the mid-1960's. Results suggest that *S. bacillaris* can survive up to 19 times the freezing and thawing cascade with no viability losses. However, no kinetic data or photosystem apparatus assessment has been performed, particularly considering the long-term growth, resulting in a lack of knowledge about this strain behavior after freeze storage. Nowadays, this microalga is usually cryopreserved by several microalgal collection, but no data have been published. However, to the best of the authors' knowledge, the overall monitoring during growth of cultures after freezing/thawing protocols was hardly investigated. The most common method of assessing the viability of a cryopreserved microalgae culture is based on the use of vital dyes such as Trypan blue, which can only penetrate severely damaged cells [35]. However, this method does not provide information on the metabolic state of the cells after recovery from ultra-low temperatures.

In this study, short-term cryopreservation of *S. bacillaris* at −80˚C has been assessed. Different pre-freezing treatments with cryoprotectants, and two thawing methods were compared by measuring viability recovery and assessing growth kinetic of *S. bacillaris* immediately after thawing and after a series batch cultivation cycles, and a new kinetic variable has been set up to this aim. The evaluation of growth and photosynthetic rate kinetics of microalgal cultures after cryopreservation is proposed here as a more effective method to verify not only the viability of a culture, but also to evaluate hidden metabolic cell damage.

2. Materials and Methods

2.1. *Stichococcus bacillaris* Nägeli and Culture Conditions

Stichococcus bacillaris Nägeli (Trebouxiophyceae, Chlorophyta) (ACUF_102) has been retrieved from The Algal Collection at the University of Naples Federico II (ACUF). The autotrophic cultivation has been carried out under axenic conditions and according to the operating condition suggested by ACUF instructions (http://www.acuf.net/). The cultivation takes place in a liquid batch system (50 mL operating volume flasks) with Bold's Basal medium (BBM) with a gentle mixing, a 12/12 h light/dark photoperiod and the light intensity set at 80 $\mu mol_{Ph} \cdot m^{-2} \cdot s^{-1}$ (measured at lid height and provided by fluorescence tubes, L36W/865 Lumilux® Cool Daylight, Osram GmbH, Germany) white LED lamps.

Temperature has been kept constant at 25˚C ± 1˚C.

2.1.1. Cultivation Monitoring

Biomass concentration was monitored daily and spectrophotometrically measured (ONDA spectrophotometer) at a wavelength of 550 nm. A calibration curve was established to determine the linear coefficient between optical density and mass concentration. In each growth test, cultures were inoculated with fixed initial cell concentrations, and monitored until reaching the steady state of the growth phase.

2.2. Cryopreservation Strategies

2.2.1. Freezing Treatments

Three different treatments were evaluated to determine the optimal preservation conditions for *S. bacillaris*. During the first growth cycle, cells were harvested in their exponential phase (approximately 5×10^7 cells·mL^{-1}) and cryopreserved using the following strategies. Microalgae samples without cryoprotective agents (CPAs) acted as a cryopreserved untreated control (untreated). A second treatment (D) involved the addition of DMSO (Sigma-Aldrich, Saint Louis, USA) to microalgal sample, reaching a final concentration of 5% (v/v) DMSO in culture medium according to the range reported by [27]. The third evaluation (DG) was prepared by adding two cryoprotective agents to the microalgal sample: DMSO at a concentration of 5% (v/v) in the culture medium, and glycerol (AppliChem GmbH, Darmstadt, Germany) at a concentration of 5% (v/v) in the culture medium. Cryoprotective solutions were sterilized through a 0.22 µm membrane filter (Ministart, Sartorius Stedim Biotech GmbH, Germany). Each treatment strategy was prepared dispensing microalgal sample up to a final concentration of 5×10^6 cells·mL^{-1} into cryovials similar to what suggested by [36]. All treatments (conducted in triplicates) were then placed in a Mr. Frosty™ (Nalgene NUNC International, USA) according to manufacturer's instructions, with the addition of 250 mL of isopropanol. After a minimum of 4 hours, the vials were quickly moved to a final box container to storage samples at the same temperature. The frozen samples were thawed after 24 hours and 1 month.

2.2.2. Thawing Methods

After storage, all frozen samples were thawed with two different methods through several steps. The first method, known as "sudden thawing" (Stw), involved an immediate thaw. Cryovials containing the frozen microalgal cultures were removed from the −80˚C storage and then immediately placed in floating holders immersed into a 35˚C water bath for 3 minutes at dark to let ice melt. Thus, samples were incubated for 1 hour in darkness to minimize any photooxidative damage [26] [28]. Afterward, the cryovials were centrifuged at 10.000 rpm for 20 minutes. The supernatant was discarded to remove any CPAs that might be toxic for thawed cells. Anyway, also untreated preserved control was washed to compare the procedure. The fresh medium was used to resuspend the samples and

used as inocula for growth tests as reported in section 2.1. To assess the long-term effect of preservation, a repeated batch cultivation has been carried out. The second method was "gradual thawing" (Gtw) which involved a gentler thawing process. Cryovials with the frozen samples were transferred from −80˚C storage to −20˚C freezer for 1 hour, and then to +4˚C storage overnight. After storage, the samples were centrifugated and processed according to the steps described in the "sudden thawing" method above.

2.3. Viability and Photosynthetic Assessment

To evaluate cultures viability and perform photosynthetic efficiency, both the unpreserved (control), and cryopreserved cultures have been harvested in their exponential phase. The following viability tests have been performed [17].

2.3.1. Trypan Blue Assay, and Chlorophyll *a* Autofluorescence Measurement

Cell cultures viability and morphology were assessed by a Typan blue permeabilization assay [35]. The cells were mixed with Trypan blue dye (Sigma-Aldrich, Germany) at a ratio of 1:1 and incubated for 1 hour to allow for eventual dye permeabilization in the nonviable cells. Aliquot of this mixture was observed using the optical microscope approach. Additionally, the chlorophyll *a* autofluorescence of microalgal cells was inspected under the fluorescence microscope using a epifluorescence microscope Nikon Eclipse E800 [37]. Specifically, these analyses were carried out immediately after thawing.

2.3.2. Cell Cultures Recovery

"Recovery from preservation" (RecP) has been assessed as a "ad hoc" kinetic variable. Briefly, culture concentrations were monitored up to the steady state of growth, resulting in growth curves for each freezing/thawing strategy. Thus, the overall biomass growth rate (μ_{ov}), the growth lag (φ_{lag}) and the biomass growth rate (μ_g) after the end of lag phase has been calculated according to the following equations:

$$\mu_{ov} = \left(\Delta X_{ov} / \Delta t_{ov}\right) / \overline{X} \quad (1)$$

$$\mu_g = \left(\Delta X_g / \Delta t_g\right) / \overline{X}_g \quad (2)$$

$$\varphi_{lag} = \mu_{ov} / \mu_g \quad (3)$$

where ΔX_{ov} represents the difference between biomass concentration (expressed as g/L) at the last checked point of exponential phase and the inoculum time, and Δt_{ov} is the relative difference; ΔX_g represents the difference between biomass concentration at last and the first checked points of exponential phase and Δt_g is the relative difference. Both control and preserved cultures were assessed using these parameters. Growth lag (φ_{lag}) is a kinetic parameter that is defined as the ratio between the overall and exponential specific growth rate, thus it is a variable able to assess the impact of lag phase on the overall growth time. Clearly, it's limited to [0, 1] interval: increasing φ_{lag} means an increase of the duration of the

lag phase, underlining the time need to adapt and restore cell metabolic pathway.

Specifically for the freezing samples, the growth lag was utilized to evaluate the adaptation of the culture immediately after storage. In order to compare this phase between preserved samples and control, the kinetic variable RecP was calculated with following equation:

$$\text{RecP} = \varphi_{lag\_preserved} / \varphi_{lag\_control} \cdot \quad (4)$$

RecP has been defined to assess the kinetic effect of preservation: briefly, it can range from 0 (no growth occurred for preserved cells) to 1 (preserved cell growth comparable to unpreserved one). In this interval, the higher the RecP the better is the kinetic response of cells to preservation strategy. Clearly, a RecP larger than 1 is unfeasible. The sensitivity limit of RecP definition is related to the time scale of test. Clearly, the smaller the scale, the more rigorous the result. In this work, microalgae growth temporal scale has to be considered, thus day was chosen as magnitude order since their low duplication time in autotrophy cultivation system. As reported in literature, microalgae cell concentrations are generally checked daily, thus the "day-scale" has been considered consistent with the aim of this kinetic variable.

2.3.3. PAM Fluorometry Assay

The photosynthetic efficiency of each microalgal culture was evaluated in their exponential phase using a FMS2 Hansatech PAM fluorometer. The samples were subjected to a dark adaptation of 30 minutes. Afterward, they were transferred into a 4 mL quartz glass cuvette with a magnetic micro-agitator, ensuring homogeneity during the tests [38]. Saturating light was applied to measure F_0 (minimum fluorescence yield) and F_m (maximum fluorescence yield), thus maximum quantum efficiency of photosystem II was calculated according to the following equation:

$$F_v / F_m = \left(F_m - F_0\right) / F_m \,. \quad (5)$$

A sequence of 9 increasing actinic irradiance, ranging from 36.56 μEin to 1213 μEin, was set to evaluate F_s (Fluorescence yield in the steady-state in light-adapted samples), F_0' (Minimum fluorescence yield in light-adapted samples), F_m' (Maximum fluorescence yield in light-adapted samples). Before each actinic irradiance, the samples were incubated at dark for 2 minutes. Relative electron transport rate (rETR) was calculated with following equation:

$$\text{rETR} = \left(F_m' - F_0'\right) / F_m' \cdot \text{PPFD} \quad (6)$$

where $F_m' - F_0'$ represents the PS II operating efficiency and estimates the efficiency at which light absorbed by PS II is used, and PPFD is the photosynthetically active photon flux density (mol quanta $m^{-2} \cdot s^{-1}$) [39].

2.3.4. Oxygen Evolution Measurement

The photosynthetic performance of each sample was also evaluated by measur-

ing the rate of oxygen evolution in response to varying light intensities [4]. Oxygen exchange rates were determined with an Oxygraph Clark-type oxygen electrode (Hansatech, King Lynn, UK) at 25˚C according to the sample cultivation temperature. After adaptation in darkness for 30 minutes, the samples were dispensed into device with a magnetic micro-agitator to homogenize the samples during the test. A sequence of 10 increasing light intensity, ranging from 18.8 μEin to 1388 μEin, was used to generate a light-response (P/I) curve. In this curve, P ($nmol_{Oxygen} \cdot mg_{biomass}^{-1} \cdot min^{-1}$) represents the rate of oxygen production, and I (μEin) represents the applied light intensity. From the above-mentioned plot, two critical parameters were calculated: the maximum light utilization efficiency (α) and the maximum oxygen production rate (P_{max}). α was calculated as the slope of the initial linear portion of the curve, while P_{max} was assessed as the maximum value reached during the plateau.

2.3.5. Pigments Assessment

The biomass obtained in the exponential phase was characterized in term of chlorophyll *a*, chlorophyll *b*, and total carotenoids concentrations. Pigments quantification was performed suspending 1 g_{DW}/L of biomass in pure methanol. The samples prepared were incubated at 37˚C overnight with slowly shaking in darkness. The pigments concentrations were quantified spectrophotometrically measuring the absorbance at 470 nm, 646 nm, and 663 nm, and using the following equations [40]:

$$Chl_a\left(\mathrm{mg/L}\right) = 16.72 * A_{663} - 9.16 * A_{646} \tag{7}$$

$$Chl_b\left(\mathrm{mg/L}\right) = 34.04 * A_{646} - 15.28 * A_{663} \tag{8}$$

$$Carot_{tot}\left(\mathrm{mg/L}\right) = \left(1000 * A_{470} - 1.63 * Chl_a - 104.9 * Chl_b\right)/221. \tag{9}$$

Additionally, the ratio between chlorophyll *a* and chlorophyll *b* and the ratio between photoactive pigments (chlorophyll *a* and chlorophyll *b*) and photoprotective pigments (total carotenoids) have been evaluated to assess the overall status of photosystem.

2.4. Statistical Analysis

Each test was conducted in three independent biological replicates, and the average values were reported. The datasets are expressed as mean (SD) to estimate the error.

Error propagation has been considered for all the variables involving ratio of measured variables. Each batch concentration has been considered as a single test before error calculation.

3. Results and Discussion

3.1. Effect of Cryopreservation on *Stichococcus bacillaris* Cell Morphology and Viability

Different pretreatments were compared with freezing and thawing strategies to

assess the best conditions for *Stichococcus bacillaris*. Cryopreservation methods are considered a stress condition for low temperature utilized to storage cells. Cryogenic temperature can promote ice crystal formation in the cells that have the potential to cause structural damage leading cell death or its morphology alteration: the use of cryoprotective agents can help the achievement of storage [17] [41]. The primary aim of preserving cells is to maintain their morphology [28]. The effects of short-term cryopreservation tested are illustrated in **Figure 1**, where only one representative example from each treatment is shown. As expected, frozen *S. bacillaris* cells were able to survive all the freezing and thawing strategies tested [34] [42]. Notably, the absence of Trypan blue dye uptake by the preserved cells was detected and shown in **Figure 1(a)**, and no change in cell morphology was detected by light microscopy observations comparing to the control shown in **Figure 1(c)**. Furthermore, chlorophyll autofluorescence provides a direct reflection of the photochemical system's functionality [4]. As shown in **Figure 1(b)**, the chlorophyll *a* autofluorescence signal of the preserved cells was equal to the control reported in **Figure 1(d)**. This means that the vitality of the cells is maintained even after thawing. However, cells can enter a state of dormancy where they are viable but not culturable [19]. Therefore, in addition to assess cell viability, the evaluation of cultivability must also be taken in consideration.

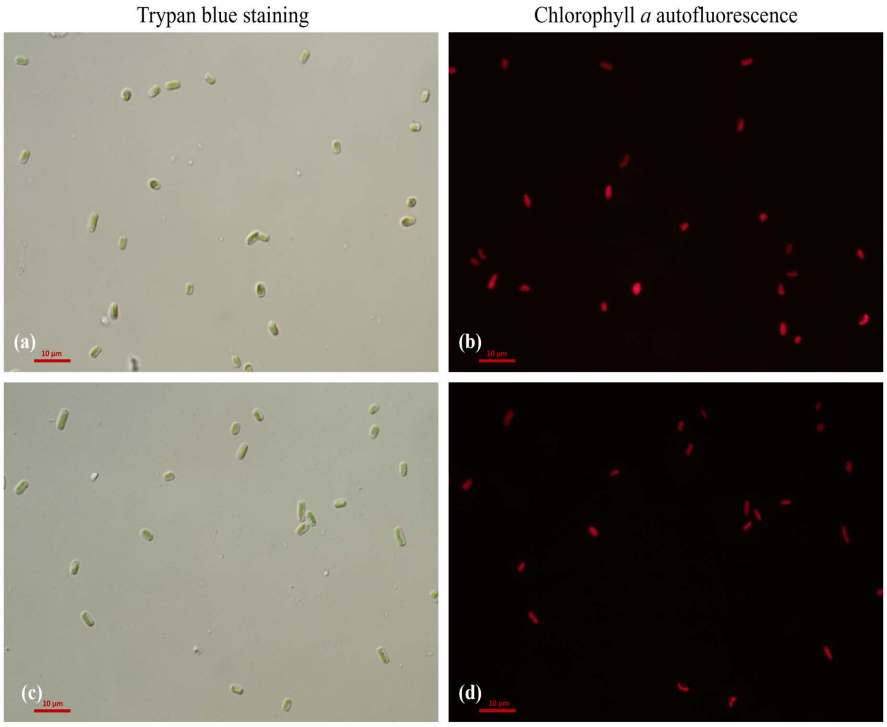

Figure 1. Light (DIC) and autofluorescence micrographs of *Stichococcus bacillaris* cells: (a-b) single case of preserved samples reported as an example, where (a) shows the absence of Trypan blue dye uptake and (b) shows the chlorophyll *a* autofluorescence signal; (c) shows the control with Trypan blue staining, (d) chlorophyll *a* autofluorescence of control. Scale bar: 10 μm.

3.2. Growth Recovery from Different Preservation Strategies

In the present study, recovery from preservation was assessed to evaluate culture adaptation both immediately after thawing and in successive batch culture growth tests. It was useful to compare different freezing treatments/thawing strategies. The recovery of cultures immediately after 24 hours and 1 month of storage is shown in **Figure 2**.

As depicted in **Figure 2(a)**, after 24 hours, the best recovery equivalent to 1 is obtained in the cultures preserved with DMSO and glycerol treatment and processed with a sudden thawing (DGStw). The same freezing treatment resulted in a 20% lower recovery when gradual thawing was employed (DGGtw).

Moreover, the freezing treatment with only DMSO and sudden thawing (DStw) showed a better recovery than all the treatments with gradual thawing. This means that an immediate thaw at 35˚C could avoid any photooxidative damage leading to immediate recovery of cultures [28]. Thus, sudden thawing can be considered the preferential strategy. It is worth noting that a satisfactory preservation recovery (20% less) was found for the cryopreserved untreated sample (U) with both thawing methods. Although only the growth retardation of DGStw is similar to the control, other cultures show a significant recovery, proving that it is possible to avoid the use of cryoprotectants for this short-term preservation, according to another study on different microalgae reported by [25].

Similar trends were observed after 1 month of storage as shown in **Figure 2(b)**: although none of the preserved samples achieved uniform recovery, and the cryopreserved untreated controls obtained approximately 20% lower recovery, the addition of cryoprotectants results in a greater recovery in both the sudden and thawing methods. Thus, for this storage time there is no significant difference between the two thawing methods, but there is an advantage using the cryoprotective agents. In particular, viability levels assessed on the basis of recovery from storage are, as expected, slightly reduced from 24 hours to 1 month of storage, suggesting that recovery is dependent on storage time. Kapoore *et al.* [23] showed that the viability level of *Chlorella vulgaris*, in this case expressed as colony formation on agar, decreased by 20% from 24 hours storage to 1 month and then 4 months storage at −80˚C. A more detailed study of the immediate effects of cryopreservation strategies is required to evaluate the long-term preservation of *Stichococcus bacillaris*.

Therefore, the recovery from preservation was also estimated in consecutive batch cultures for all strategies. All cultures obtained a recovery equivalent to 1 (data not shown). This means cryoprotective agents can increase the kinetic recovery of preserved cells in terms of reduction of lag phase during the first batch cultivation tests immediately after the thawing. However, all preserved cell cultures are always able to restore their growth kinetic with a complete recovery. It is mandatory to underline the strategy optimization should be proved considering other parameters, including cost analysis for materials, and the consequences

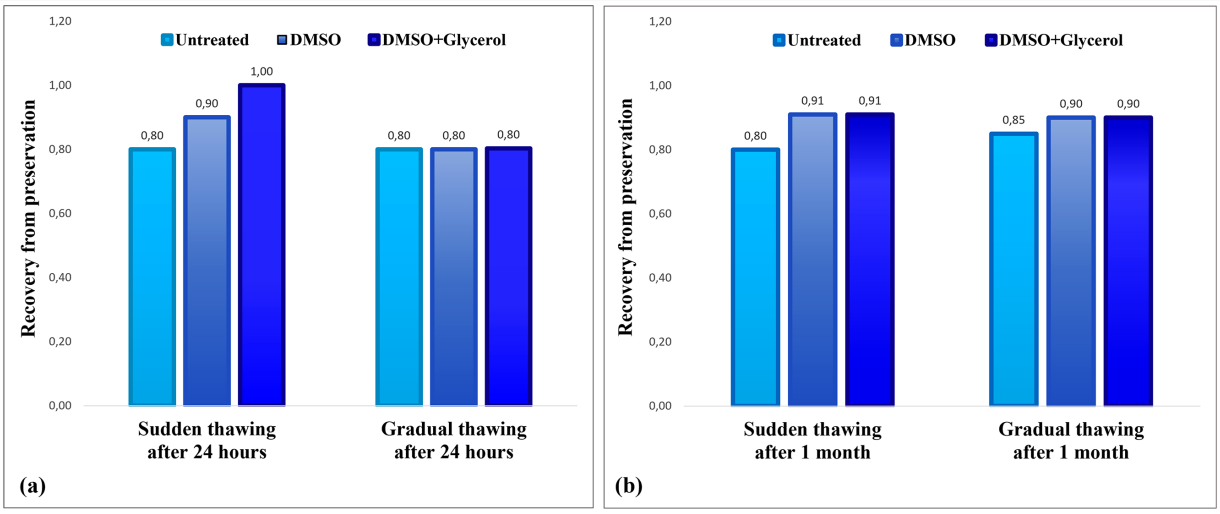

Figure 2. Results of growth recovery from different preservation strategies of *Sticochoccus bacillaris*: (a) recovery after 24 hours of storage, (b) recovery after 1 month of storage. Untreated: cryopreserved untreated control sample; DMSO: sample cryopreserved using dimethyl sulfoxide; DMSO + Glycerol: sample cryopreserved using dimethyl sulfoxide and glycerol.

of cryoprotective agents' addition over time. In conclusion, since the repeated batch cultivation results in a uniform recovery for each preservation strategy, the untreated sample can be considered a viable option for freeze storage up to 1 month.

3.3. Photosynthetic Efficiency and Oxygen Production Rate Reestablishing

In photosynthesis, light energy is captured in two photoreactions carried out by two pigment-protein complexes: Photosystem II (PS II) and Photosystem I (PS I), which operate in series and are linked by an electron chain. The maximum quantum efficiency of PSII (F_v/F_m) is used as a convenient estimate of the photosystem efficiency and is shown in **Figure 3**. All the various samples subjected to different freezing and thawing treatments exhibited F_v/F_m values comparable to the control. Data show that there is no significant difference between different storage times. In addition, the F_v/F_m values range from 0.7 to 0.8 in accordance with the ratio value in dark-adapted green microalgae reported by [4]. In addition, supporting data was provided by [25], who reported a functional recovery of PS II in *Lobosphaera incisa* after freezing storage at −80˚C, after three days from thawing, pairing present work results since PSII assay has been performed during exponential phase of batch culture. The photosynthetic capacity to utilize absorbed light energy through electron transport rate can be calculated as rETR (relative electron transport rate) as a function of increasing light intensity. Similar to the F_v/F_m results, all rETR values obtained from different treated samples are comparable and similar to the control (data not shown). These results demonstrated the ability of the preserved cultures to perform photoreactions without significant damage and all cryopreservation strategies had no effect on photosynthetic efficiency up to 1 month of storage.

The evaluation of the oxygen production rate, assessed by the estimation of α and P_{max} values reported in Table 1, provides further confirmation of these results. Both after 24 hours and after 1 month of storage, the overall maximum light utilization efficiency is comparable in all samples analyzed. Similarly, the cell cultures reach a comparable value of the maximum oxygen production rate in all stored samples. Although the oxygen evolution rate and photosynthetic efficiency was unsensitive to different preservation strategy, their correlation is not necessarily granted: in fact, a decrease in maximum oxygen rate can be paired with a constant electron transfer rate when the damage to photosystem is not directly related to the electron transfer, as clearly described by [36]. Clearly, an electron transfer rate drop surely results in a lower oxygen evolution. In this work, the oxygen evolution has been considered comparable and consistent with the promising results of recovery from preservation assessment (Section 3.2).

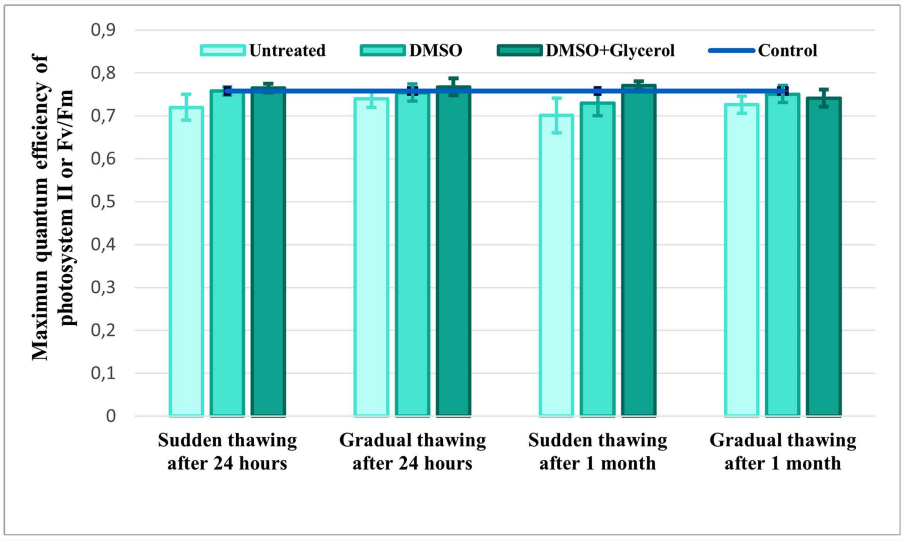

Figure 3. Maximum quantum efficiency of Photosystem II or F_v/F_m of different preservation strategies both after 24 hours and 1 month of storage. Untreated: cryopreserved untreated control sample; DMSO: sample cryopreserved using dimethyl sulfoxide; DMSO + Glycerol: sample cryopreserved using dimethyl sulfoxide and glycerol.

Table 1. Evaluation of oxygen production rate.

Preservation time	Sample	Oxygen evolution parameters	
		The maximum light utilization efficiency (α)	The maximum oxygen production rate (P_{max})
-	Control	0.13	54.22
24 hours	Untreated Stw	0.17	47.04
	DMSO Stw	0.22	59.16
	DMSO + Glycerol Stw	0.15	44.09
	Untreated Gtw	0.19	57.45
	DMSO Gtw	0.20	55.86
	DMSO + Glycerol Gtw	0.26	65.09

Continued

	Untreated Stw	0.16	50.57
	DMSO Stw	0.14	36.84
1 month	DMSO + Glycerol Stw	0.14	50.32
	Untreated Gtw	0.14	52.89
	DMSO Gtw	0.17	46.74
	DMSO + Glycerol Gtw	0.17	47.65

3.4. Pigment Mass Fraction Results

A further evaluation of the condition of the preserved microalgal cultures was assessed by pigments analysis. The results of the pigment mass fraction of the biomass are shown in Figure 4. As depicted in Figure 4(a), there were no substantial changes observed in carotenoids and chlorophyll *a* content after 24 hours of storage for both the untreated preserved sample and the sample thawed using both methods. Similarly, the chlorophyll *b* content was unaffected in the untreated sample thawed by sudden methods (UStw) and slightly increased in the same sample thawed by gradual methods (UGtw). In general, cryopreservation strategies without cryoprotectants did not alter pigments content. However, a slight increase in chlorophyll *a* and a proportional increase in chlorophyll *b* were observed in both DStw and DGtw. No significant change was observed in carotenoid production. This indicates that the utilization of DMSO as a cryoprotective agent results in an increase in total chlorophyll content, while the thawing method does not impact the chlorophyll content. Instead, when both cryoprotectants, DMSO and glycerol, are used, the difference in the thawing method becomes relevant: sudden thawing leads to an increase in chlorophyll *b*, whereas gradual thawing leads to a 50% increase in chlorophyll *a* and a decrease in chlorophyll *b*.

Figure 4(b) shows that after 1 month of storage, chlorophyll *a* content was unaffected in all preserved cultures, except in DGtw where a slight increase was observed. Conversely, chlorophyll *b* content was drastically reduced in all cultures. In particular, the chlorophyll *b* content in DGGtw was the same after 24 hours and 1 month of storage. The carotenoid content was not affected in any of the cases analyzed.

Obtained results in pigments content could be related to the growth kinetic recovery discussed in the Section 3.2. It is worth noting that the observed decrease in chlorophyll *b* content was obtained in the preserved samples that showing a growth retardation immediately after thawing with a recovery from preservation (RecP) less than 1. These results suggest a correlation between the chlorophyll *b* content and the kinetic performance. Thus, detailed investigations are mandatory to assess the significance of this correlation.

A more detailed effect on pigments is shown in Figure 4(c) and Figure 4(d), where the ratio between chlorophyll *a* and chlorophyll *b* (Chl *a*/Chl *b*) and the ratio of total chlorophyll (Chl Tot) to carotenoids (Carot) are reported. Even if

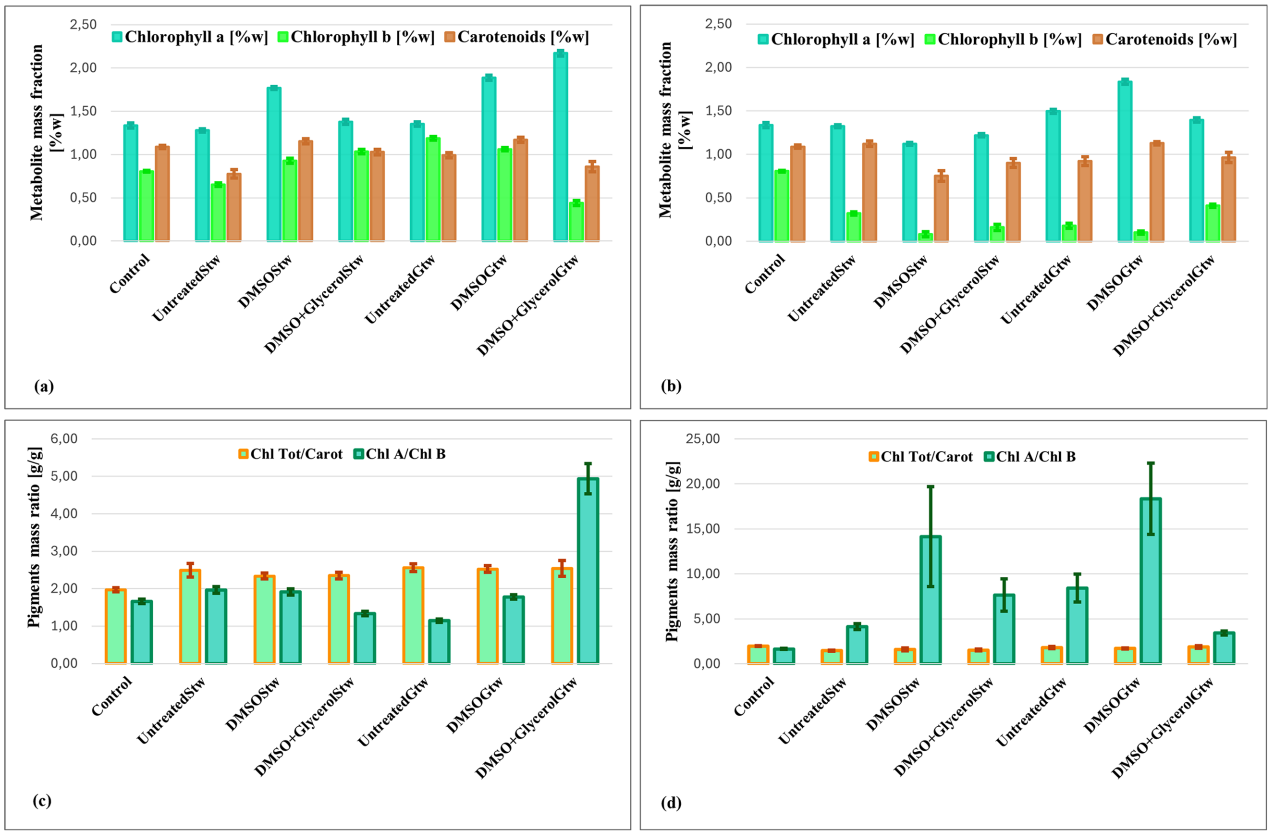

Figure 4. Results of the pigments mass fraction of the biomass: pigments mass fraction [%w] of the biomass after 24 hours of storage (a), and after 1 month of storage (b), total chlorophylls to carotenoids ratio (Chl Tot/Carot) and chlorophyll *a* to chlorophyll *b* ratio (Chl A/Chl B) after 24 hours of storage (c), and after 1 month of storage (d).

after 24 hours of storage the Chl *a*/Chl *b* ratio is comparable, except for DGGtw (drastically higher), both for the increase of chlorophyll *a* and the decrease of chlorophyll *b* as reported above, after 1 month of storage the ratio between chlorophyll *a* and chlorophyll *b* is increased in all the samples analyzed, especially when using only DMSO as cryoprotectant. In addition, for all treatments and storage times, the ratio of photoactive to protective pigment is statistically comparable to the control, suggesting that the overall status of the photosystem is not affected, in line with the results of photosynthetic performance reported in Section 3.3.

However, it is important to emphasize that the analyses conducted in this study to assess the photosynthetic apparatus efficiency were performed during the exponential phase of culture growth following thawing. This implies that the difference in chlorophyll production has a lasting impact on the preserved cells. Supporting data for the increase in the chlorophyll *a* content were presented by [25], who reported an increase in the chlorophyll content after thawing during the growth of the cultures. In higher plants, chlorophyll *b* acts as an antenna pigment and plays a key role in protein stabilization of the antenna complex, regulating its degradation. In addition, chlorophyll *b* participates in the protection of grana stack membranes from high light intensities [43].

Pioneering studies on *Chlorella* cells led to the hypothesis that energy requirements in the form of ATP are higher when chlorophyll *b* concentrations are low. More recently, Perrine *et al.* [44] reported that a partial reduction in chlorophyll *b* levels in the green alga *Chlamydomonas reinhardtii* resulted in a reduction in the size of the peripheral light-harvesting antennae, allowing increased photosynthetic efficiency at high light intensities. The observed decrease in chlorophyll *b* in *S. bacillaris* after recovery from 1 month of cryopreservation could be interpreted as an early signal of a change in the structure of the peripheral antenna protein complexes, whose impact on cell metabolism needs to be assessed.

4. Conclusion

Sticochoccus bacillaris has historically been preserved at ultra-low temperatures (−196˚C). However, there is currently no standardized cryopreservation procedure for cultures and poor evaluation related to preservation of the photosystem apparatus. The present study confirmed *S. bacillaris* as one of the best candidates for short-term preservation. Importantly, preserving it at −80˚C storage proves to be a viable strategy. The results obtained in this study are encouraging, particularly considering the overall kinetic recovery, proving that it is possible to avoid the use of cryoprotectants at −80˚C storage. It is worth noting that immediately after thawing using DMSO and glycerol as cryoprotective agents could make advantage to recovery. However, even if the chosen preservation strategies allowed the cultures to perform photosynthesis without significant damage after 24 hours of storage, the results suggest that the overall efficiency of the photosystem was not affected after 1 month of storage, although the ratio between chlorophyll *a* and chlorophyll *b* was clearly altered. Long-term studies will be conducted to assess whether the differences found could cause chronic damage to photosystem efficiency of *S. bacillaris* cultures.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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