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Effect of Low-Temperature Preservation on Growth Characteristics of Marine Microalga *Nannochloropsis salina*

M. Muthu Abishag, M. Mohammed Faizullah and M. Kokilavani

Dr. MGR Fisheries College and Research Institute, Tamil Nadu-601 204, India
Dr. J. Jayalaithaa Fisheries University, Thalainayeru, Nagapattinam-614 712, India
E-mail: mabishag@gmail.com

Abstract: The present study aimed to preserve the microalgae, *Nannochloropsis salina* at three different temperatures (0, -4 and -20℃) following standard protocols and assess their viability and growth characteristics upon thawing. The algal cells were preserved in Conway medium following seven different treatments: 0℃ without addition of cryoprotectant, -4℃ without cryoprotectant, -4℃ with 5 and 10% Me_2SO and - 20℃ with 5 and 10% Me_2SO along with a control at room temperature (24℃) for 5 days. Upon air thawing, the cells were sub-cultured at optimum conditions and were analyzed for the growth characteristics, viz., biomass, specific growth rate, cell density and optical density for 5 days. Among the treatments, the sample preserved at -4℃ without addition of any cryoprotectant exhibited better performance in terms of biomass (0.0165 g/ml), optical density (0.0265) and cell density (4.78 x 10^5 cells/ml). Similarly, the cells preserved at -20℃ with 10% cryoprotectant exhibited poor growth performance in terms of cell density (1.86 x 10^5 cells/ml) and biomass (0.0085 g/ml) which may be attributed to the toxicity of cryoprotectants. Thus, *Nannochloropsis salina* can be preserved at -4℃ without adding any cryoprotectant. This eliminates the need to maintain continuous sub-cultures in aqua hatcheries, which is laborious and costly.

Keywords: Cryoprotectant toxicity, Thawing, Dimethyl sulphoxide, Conway medium, Microalgal culture

Aquaculture, the fastest-growing food production sector, heavily relies on microalgae due to its nutritional benefits, particularly for larval stages of fish, molluscs, and crustaceans in hatcheries. Key microalgae used in aquaculture include *Nannochloropsis* sp., *Chlorella* sp., *Chaetoceros* sp., *Tetraselmis* sp., *Scenedesmus* sp., *Pavlova* sp., *Phaeodactylum* sp., *Skeletonema* sp., and *Thalassiosira* sp.(Hemaiswarya et al., 2011 , Sirakov et al., 2015). Among these, *Nannochloropsis* spp. stands out for its small size (2-4 µm diameter, subspherical or cylindrical), high growth rates, and resilience to environmental fluctuations in temperature, light, and nutrient levels. *Nannochloropsis* spp. belongs to the group, Eustigmatophyceae and are highly valued in aquaculture for their nutritional profile, containing 43% protein, 16.6% lipid, and high levels of eicosapentaenoic acid (EPA), which constitutes 16% of the total lipid content (Guimarães et al., 2021). In addition to its nutritional benefits, *Nannochloropsis* spp. exhibit high photosynthetic efficiency and a digestible cell wall composed of simple polysaccharides (63 to 119 nm in thickness) and are rich in pigments like chlorophyll, zeaxanthin, canthaxanthin, and astaxanthin (Shah et al., 2018). These properties make *Nannochloropsis* spp. an ideal candidate as live feed and for enriching zooplankton, which can further enhance the growth of aquaculture species (Siddiqui et al., 2024). Algae, both micro and macroalgae, are also known for their rich source of bioactive compounds for biotechnological interventions (Siddhnath and Kaur 2023).

The feeding of larval stages of aquatic organisms in hatcheries heavily relies on a steady supply of microalgal starter cultures, which can be sourced from commercial suppliers, government labs, universities, or directly from other hatcheries. Obtaining starter cultures from other working hatcheries is common, as many operators willingly share cultures with fellow culturists. Once acquired, these starter cultures are used to inoculate new cultures, creating a stock culture that is then serially sub-cultured. However, this conventional method demands significant space, substantial growth media, and incurs high labor costs. Additionally, it poses ongoing challenges with contamination and genetic drift, which may alter the desired characteristics of the algal strains over repeated sub-culturing (Helliwell et al., 2011, Berge et al., 2012). An effective alternative is low-temperature preservation. Over the past years, different protocols and cryoprotective additives (CPAs) have been tested in order to increase the post-thaw survival rate of microalgae (Abreu et al., 2012, Chellappan et al., 2020). There is no universal protocol for the cryopreservation of algae, which is not surprising considering the substantial heterogeneity in morphology, physiology, and ecology. Hatcheries generally have access to freezer storage for chemicals and feeds, making low-temperature storage an accessible option. This study, therefore, aims to develop a cost-effective method to store algal cultures at low temperatures, allowing hatcheries to thaw and sub-culture algae on demand, reducing resource use and preserving the integrity of algal strains.

MATERIAL AND METHODS

Species for study: The microalgal species used for the study, *Nannochloropsis salina* was obtained from the algal culture laboratory, Seabass Hatchery Unit, Rajiv Gandhi Centre for Aquaculture, Sirkali. Conway medium was used for the invitro culture of *Nannochloropsis salina* (Table 1). All glassware used in the study were sterilized in autoclave at 121℃ (15 lbs pressure) for 15 minutes. Ten milliliters of collected samples were transferred aseptically to the conical flasks (ml) containing the respective growth media. The experiments were performed at 22°C and the cultures were illuminated underneath by cool-white fluorescent lamps with a Photosynthetically Active Radiation (PAR) of 50 μmol photons $m^{-2}s^{-1}$ under a 12L: 12D regime in growth media prepared using 33 ppt artificial seawater (Table 2). The distilled water was autoclaved at 121°C for 15 minutes, prior to the preparation of artificial seawater to avoid contamination. The cultures were manually shaken twice daily as no agitation through aeration was provided. The culture was used as an inoculum and stock culture for further studies.

Preservation of the microalgal cells: Dimethyl sulfoxide (Me_2SO) was used as cryoprotectant in this study. The cryoprotectant was mixed with distilled water at twice the desired final concentration. For preparing 5% Me_2SO, 10 ml of Me_2SO was mixed with 10 ml of water and finally made up to 100 ml. In the same way 10% Me_2SO solution was prepared.

Preservation at 0°C: For preservation of the sample at 0°C, 2ml of stock culture without any cryoprotectant was taken in vials in duplicate and were stored in refrigerator for 5days. The preservation of samples at -4°C was carried out with and without the addition of cryoprotectant. One portion of the sample was stored at -4℃ without the addition of any cryoprotectant. For preservation using cryoprotectant, 0.1 ml of 5% Me_2SO was added with 1.9 ml of stock culture in 2 vials and 0.1 ml of 10% Me_2SO was added with 1.9 ml of stock culture in separate vials. All the vials were stored at -4°C for 5days. For preservation at -20°C, 0.1 ml of 5% Me_2SO along with 1.9 ml of stock culture taken in 2 vials and 0.1 ml of 10% Me_2SO was added with 1.9 ml of stock culture in separate vials. The vials were rapid frozen using 'Mr. Frosty' Freezing container (Nalgene®, Nalge Nunc International) containing isopropyl alcohol, previously cooled to 4°C, at a freezing rate of-1℃ min. The frozen vials were stored at -20°C for 5 days.

Thawing and post-thaw evaluation: After 5 days of storage, the vials were taken and air-thawed at room temperature (24°C). After thawing, the contents of the vials were diluted by stepwise addition of 100 ml of growth medium (Conway medium) and the cultures were incubated at 24°C (at 1000 lux) along with control (stock culture) and their growth characteristics viz., biomass, cell density, Specific Growth Rate and optical density (OD) were analyzed daily for 5 days.

Biomass: Biomass was estimated using 2 ml microalgal samples filtered through pre-combusted (100°C, 4 h) and pre-weighed glass fibre filters (Advantec, Japan). After filtration, algal samples were rinsed with 2 ml of 0.5 M ammonium formate. The filtrates were dried at 100°C for 4 h, cooled in a desiccator and then weighed. The dry biomass concentration in the culture was calculated by dividing the difference between the weights of the dried filter paper (after and before filtration) by the filtered volume (Lavens and Sorgeloos 1996).

Cell density: Cell numbers were determined daily by placing an aliquot of well-mixed culture suspension on a hemocytometer (Neubauer Improved Assistant, Germany).

Table1. Chemical composition of Conway medium

Chemical name	Quantity (g/L)
Solution A	
Potassium nitrate(KNO_3)	10
Sodium Di-hydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	2
EDT Adi sodium salt(Na_2EDTA)	4.5
Boric acid (H_3BO_3)	3.34
Ferric chloride($FeCl_3$)	0.13
Manganese chloride($MnCl_2$)	0.036
Solution B	
Zinc chloride($ZnCl_2$)	0.42
Cobalt chloride ($CoCl_2.6H_2O$)	0.4
Copper sulphate ($CuSo_4.5H_2O$)	0.4
Ammonium molybdate	0.18
Con. Hydrochloric acid	2ml
Solution C	
VitaminB_1	0.002
Vitamin B_{12}	0.001

To prepare Conway medium, 1ml of solution A, 0.5 ml of solution B and 0.1 ml of solution C were mixed with 1 litre of seawater

Table 2. Chemical composition of artificial seawater

Compound	Quantity (g/L)
Sodium chloride (NaCl)	28.32
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	5.48
Magnesium sulphate($MgSo_4$)	3.60
Calcium chloride ($CaCl_2.2H_2O$)	1.11
Potassium chloride (KCl)	0.77
Sodium bicarbonate	0.18

The cell number in the culture was calculated by dividing the number of cells counted by the volume and the dilution. The cell growth was measured by Neubauer counting chamber and the total number of cells were calculated using the following formula:

Total cell count (per ml) = Average no. of cells in each chamber x 10^4

Optical density: The optical density (OD) values for all cultures were determined daily using a UV-Vis spectrophotometer (Systronics, India). The wavelength used was 680 nm.

Specific growth rate: The specific growth rate (SGR) of microalgae was calculated by the following equation:

$$SGR/day = \ln (X_2/X_1)/t_2-t_1$$

X_1 is the biomass concentration at the beginning of the selected time interval;

X_2 is biomass concentration at the end of the selected time interval;

t_2-t_1 is the selected time (in days) for the determination of biomass of microalgal species.

Statistical analysis: Analysis of Variance (ANOVA) and the post-hoc analysis using Tukey's Honestly Significant Difference (HSD) was done using SPSS version 26.

RESULTS AND DISCUSSION

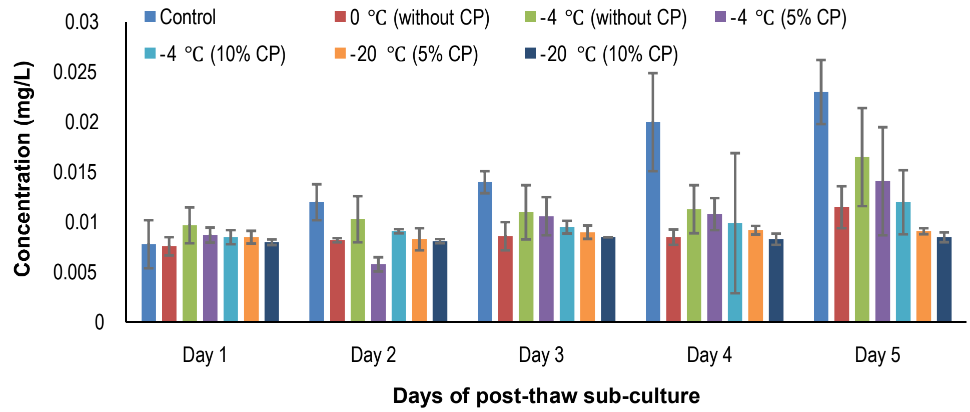
Biomass and specific growth rate: The increase in biomass observed across all treatments with continued sub-culturing suggests that the microalgal cells remained viable after low-temperature preservation and successfully resumed growth post-thawing (Fig. 1). This rise in biomass is indicative of the cells' ability to recover and proliferate following cryopreservation. After five days, the sample preserved at -4°C without any cryoprotectant demonstrated significantly highest biomass concentration (0.0165 g/L), which suggests that the absence of cryoprotectant might have mitigated potential stressors, allowing for optimal recovery and growth followed by biomass in cells preserved at -4°C with 5% cryoprotectant (0.141 g/mL). Similarly, the cells preserved at -4°C exhibited the highest specific growth rate (0.003 - 0.005 g/mL/day) at the 4th and 5th days of post-thaw sub culture (Fig. 2). This result implies that a moderate cryoprotectant concentration may have supported cell viability while reducing damage from freeze-thaw cycles, aligning with findings from previous studies that suggest a balanced cryoprotectant concentration is often beneficial for post-thaw viability and growth (Hubálek 2003). The lowest biomass concentration was observed in cells preserved at -20°C with 5% cryoprotectant (0.0085 g/mL) which may be due to increased cryoprotectant toxicity and potential osmotic stress, particularly at higher concentrations. Chellappan et al. (2020) also reported that elevated cryoprotectant levels could reduce post-preservation growth rates, as evidenced in diatoms that showed higher growth rates after storage with reduced cryoprotectant exposure in liquid nitrogen vapor phase.

Optical density: The differences in optical density (OD) among the samples preserved under various conditions highlight the impact of cryoprotectant concentration on cell preservation and, consequently, biomass retention (Fig. 3). The highest OD was in the sample preserved at -4°C without any cryoprotectant (0.0265) indicates that the absence of cryoprotectant may have minimized potential osmotic or metabolic stress on the cells. This preservation method possibly allowed for better maintenance of cellular integrity, leading to higher biomass retention as seen in the OD measurement. This was followed by samples preserved with 5% cryoprotectant at -4°C (0.0255). This suggests that a moderate cryoprotectant concentration might offer a balance between protecting cells from freeze-thaw damage and minimizing osmotic stress (Prieto-Guevara et al., 2023). Cryoprotectants are known to stabilize cell membranes and protect intracellular components, yet higher concentrations can sometimes induce osmotic imbalances or stress, which could potentially harm cells. Moreover, Me_2SO, usually, at a

Table 3. Variations in the cell density of *Nannochloropsis salina* upon thawing and sub-culturing

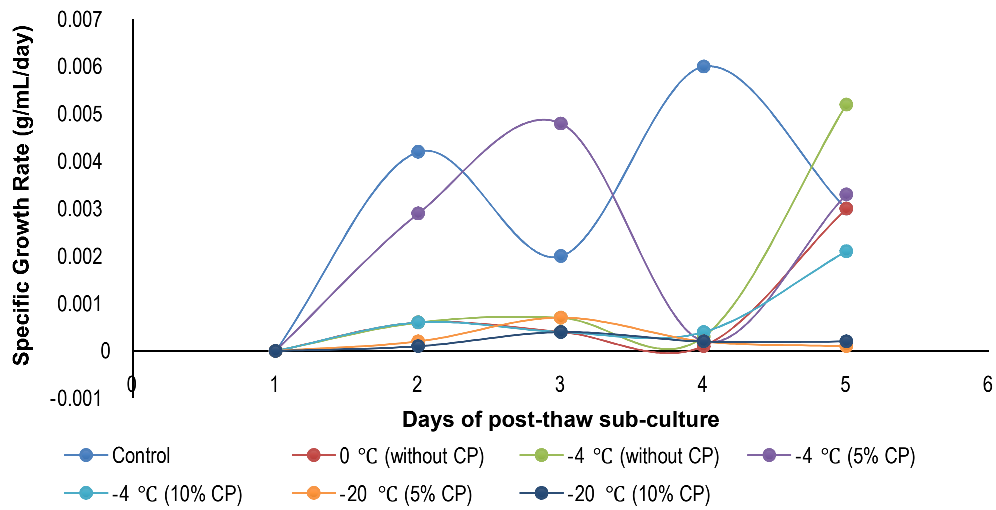
Treatment	Cell density (x10^5 cells/ml)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Control	2.02 ±0.016^b	2.14±0.0014^{bc}	2.56±0.02^{cd}	2.98±0.022^b	3.52±0.024^{bc}
0℃ (without CP)	1.65±0.002^b	1.97±0.004^c	2.22±0.0055^{cd}	2.55±0.0065^{bc}	2.58±0.01^c
-4℃ (without CP)	2.22±0.012^b	3.83±0.0155^a	4.47±0.0195^a	4.7±0.021^a	4.78±0.0265^a
-4℃ (5% CP)	3.27±0.075^a	3.11±0.0038^{ab}	3.75±0.015^{ab}	4.16±0.0175^a	4.19±0.0255^{ab}
-4℃ (10% CP)	2.55±0.055^{ab}	2.67±0.0075^b	2.95±0.0014^{bc}	3.35±0.0124^b	3.43±0.0028^{bc}
-20℃ (5% CP)	1.98±0.004^b	2.22±0.0044^{bc}	2.45±0.007^{cd}	2.6±0.008^{bc}	2.7±0.012^c
-20℃ (10% CP)	1.57±0.0014^b	1.71±0.0035^c	1.8±0.0035^d	1.82±0.0045^c	1.86±0.008^c

The values are expressed in Mean ± Standard deviation. Mean values having different superscripts on the same column are statistically different ($p < 0.05$)



The values are expressed in Mean ± Standard deviation. Error bars indicate standard deviation Mean values having different superscripts on the same day are statistically different ($p < 0.05$). The percentages in the paratheses of the legend represent the concentration of cryoprotectant used in the treatment

Fig. 1. Variations in the biomass of *Nannochloropsis salina* upon thawing and sub-culture



The percentages in the paratheses of the legend represent the concentration of cryoprotectant used in the treatment

Fig. 2. Variations in the post-thaw specific growth rate of *Nannochloropsis salina* subjected to various storage temperatures

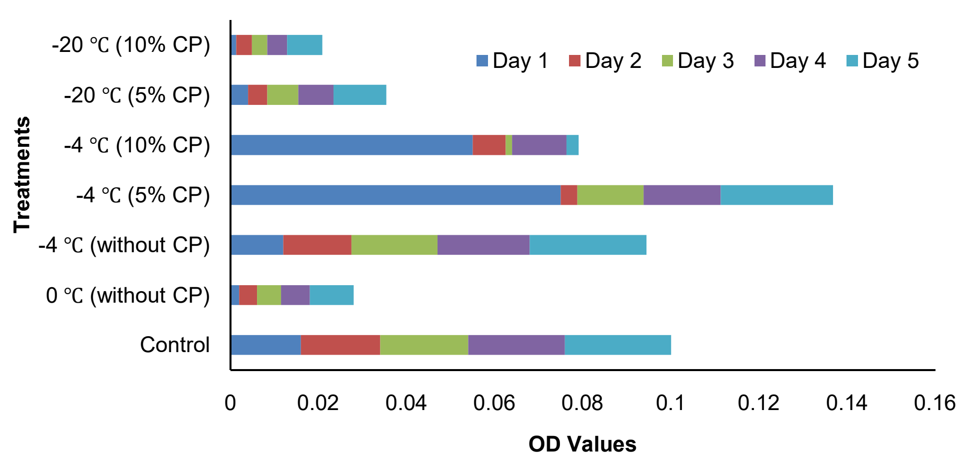


Fig. 3. Variations in the optical density of *Nannochloropsissalina* upon thawing and sub-culture

concentration of 5-10% has proved to be the most effective cryoprotective agent for a wide range of microalgal classes due to its low molecular weight, permeability, and free radical scavenging activity (Thomashow 1999). The reduced OD with 10% cryoprotectant likely reflects such a stress response, possibly leading to compromised cell integrity and reduced biomass retention. The lowest OD observed in the *Nannochloropsis salina* preserved with 10% cryoprotectant implies that this higher concentration may have negatively affected cell viability. *Nannochloropsis salina* might be sensitive to higher cryoprotectant concentrations, which could lead to cell shrinkage, membrane damage, or other structural issues. This is in alignment with the lower biomass values obtained, suggesting that while cryoprotectants provide a degree of protection, the concentration must be carefully optimized. *Nannochloropsis* sp. and other microalgal species have also indicated that low to moderate cryoprotectant concentrations (typically 5%) often preserve cells effectively by reducing ice-induced mechanical damage without introducing excessive osmotic stress (Abreu et al 2012).

Cell density: The cell density of the samples increases with the days of sub-culture. The highest cell density (4.78 x 10^5 cells/ml) was observed in samples preserved at -4°C without cryoprotectant, with statistical significance. The absence of cryoprotectants can sometimes be favourable, as cryoprotectants, while protective, may introduce osmotic or toxic stresses at certain concentrations (Hubálek 2003). The cells in this treatment were able to resume growth rapidly, leading to high post-thaw cell densities. The followed by in samples preserved at -4°C with 5% cryoprotectant. This treatment likely balanced the protective benefits of the cryoprotectant with minimal osmotic stress, promoting cell recovery and growth, as reflected in consistent biomass and OD values (Abishag et al 2025). The lowest cell density was recorded in samples preserved at -20°C with 10% cryoprotectant (1.86 x 10^5 cells/ml). This lower density may be due to the toxicity or osmotic stress introduced by the higher cryoprotectant concentration and the harsher preservation temperature, which can lead to membrane disruption and reduced cell recovery rates (Prieto-Guevara et al., 2023). Higher concentrations of cryoprotectants can create hyperosmotic environments, which may harm cells, especially at lower temperatures where cryoprotectant toxicity can be exacerbated. The findings highlight the importance of optimizing cryoprotectant concentration and storage temperature to ensure high post-thaw viability and recovery in microalgal cultures, particularly in species sensitive to osmotic stress.

CONCLUSION

The results demonstrate that *Nannochloropsis salina* preserved at -4°C without any cryoprotectant exhibited superior growth performance, as indicated by higher biomass, cell density, and optical density values. In contrast, cells preserved at -20°C with 10% cryoprotectant showed poorer growth performance, likely due to the cryoprotectant's toxicity at this concentration. These findings underscore the critical role of optimizing both cryoprotectant concentration and storage temperature to maintain high post-thaw viability and recovery in microalgal cultures, particularly in species sensitive to osmotic stress. Additionally, integrating gene expression analysis and omics approaches could provide valuable insights into the effects of preservation on the production of bioactive compounds such as pigments and antioxidants, with applications in aquaculture and biotechnology.

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