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Research Article

Development of Alternative Technology for the Long-term Storage of Microalgal Stock Culture in Fish Hatcheries

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ABSTRACT

Maintenance of algal culture faces several problems associated with climate change, contaminations, equipment failures, power failures, unexplained crashes, poor lab facilities. Microalgae are traditionally preserved by serial subculture methods that are laborious, costly, and high risk of culture contamination. While unique characteristics may not be durably maintained with general subculture, cryopreservation methods better prevent alterations from desired characteristics. Because of the cost-effective in replacing of liquid nitrogen at every interval of time and technical person needed for the preservation. The objective is to find out an alternative and a low-cost technology for the preservation of microalgae Stock culture. The microalgae *Nannochloropsis salina*, *Chlorella volutis*, *Cheatoceros gracilis*, *Dunaliella* sp. and *Amphora* sp., were preserved using common cryoprotectants (methanol, DMSO, ethylene glycol and glycerol) for 6 months at –196˚C and –20˚C. The viabilities of the microalgae were assessed after thawing, and cell counts were measured. The preserved algae *Nannochloropsis salina*, *Chlorella volutis*, *Dunaliella* sp. and *Amphora* sp. evoked good responses with negligible changes in their survivability in 6 months incubation period when preserved at –20˚C and – 196˚C, while the *Cheatoceros gracilis* regenerate only in –196˚C but not restored in –20˚C. In this study, an alternative method for liquid nitrogen preservation has been standardized and this new method can be a boon for small level fish hatcheries and microalgal stock holders.

Keywords: Microalgae; Stock culture; Cryoprotectants; Cryopreservation; Viability; Dimethyl Sulfoxide (DMSO)

INTRODUCTION

Microalgal biotechnology is a widespread field [1,2]. A variety of compounds are obtained from the microalgae for diverse industrial applications. Some examples are dyes, antioxidants, emulsifiers and gelling used in alimentary industry [3-5], aminoacids, omega 3 and omega 6 fatty acids used for medical applications [6,7], moisturizers and sunscreen used for cosmetic production [2,3], bio combustible production [8] and environmental applications [9,10]. Microalgae are the main component of first tropic level in aquatic food chain; it is for this reason that they are used as food in aquaculture [11]. Microalgae are the important food source and feed additive in the commercial rearing of aquatic animals, especially larvae, spat of bivalve molluscs, penaeid prawn larvae and live food organisms such as rotifers which, in turn, used to rear the larvae of marine finfish and crustaceans.

The maintenance of microalgae collections based on agar is well established and it is labor intensive, costly and subject to contamination and genetic change [12]. Microalgae preserved are usually maintained by serial sub-culturing. This method is time consuming and expensive. Additionally, the risk of bacterial contamination due to repeated manipulations is high; transfer of stocks is time consuming and exhausting over long periods. Many species of microalgae do not survive periodically and routine transfer of subcultures [13-15]. The only preferred solution is cryopreservation. But the major problem in cryopreservation is cost effective so the small-scale stock holders are unaffordable for using this technique. Although studies are available on cryopreservation effect on marine algae, but work on low temperature preservation like –20°C in marine microalgae is inadequate and this technique of preservation will help the microalgae culture collection centers, small-scale algal based industries, stock holders, fish hatcheries, laboratories, Universities etc. for the long-term storage of desired microalgae without cryopreservation.

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MATERIALS AND METHODS

Collection of microalgae

Six species of microalgae like *Nannochloropsis salina*, *Chlorella volutis*, *Cheatoceros gracilis*, *Dunaliella* sp. and *Amphora* sp. were obtained from Planktology and Aquaculture Division, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam.

Cryoprotectants and their concentrations

The microalgae were preserved using standardized cryoprotectant concentrations and 30 minutes equilibration time obtained from the preliminary study. *Nannochloropsis salina* were preserved using 10% DMSO, 15% methanol, 2.5% glycerol and 12.5% ethylene glycol, *Chlorella volutis*, *Dunaliella* sp. and *Amphora* sp., were preserved with 10% DMSO, 15% methanol, 2.5% glycerol and 15% ethylene glycol and *Cheatoceros gracilis* was preserved with 10% DMSO and 10% methanol. All the chemicals used in this study were purchased from HiMedia, India.

Cryopreservation protocol

The cryoprotectants were added to distilled water or seawater twice the desired concentration. Log phase or stationary phase microalgal culture was centrifuged at 5000 rpm for 5 minutes, removed the supernatant and resuspended the pellets with the culture medium for making a cell suspension of ~10^6 cells ml^{-1}. Then 0.5 ml of cryoprotective solution and 0.5 ml algal (~10^6 cells ml^{-1}) culture were gradually added to (1:1 ratio) over the course of 30 minutes with cooling in an ice bath. Aliquots of 1 ml of the resulting suspension was dispensed and introduced into a cryogenic vial and hold for 30 minutes in an ice bath. The cryogenic vials were introduced in to the – 196° C freezer and under controlled freeze at 1^0 C min^{-1} in 45 minutes and to be kept for freezing for 30 days and six months [16].

Low temperature preservation protocol (–20°C preservation)

The cryoprotectants were added to distilled water or seawater twice the desired concentration. Log phase or stationary phase microalgal culture was centrifuged at 5000 rpm for 5 minutes, removed the supernatant and resuspended the pellets with the culture medium for making a cell suspension of ~10^6 cells ml^{-1}. Then 0.5 ml of cryoprotective solution and 0.5 ml algal (~10^6 cells ml^{-1}) culture were gradually added to (1:1 ratio) over the course of 30 minutes with cooling in an ice bath. Aliquots of 1 ml of the resulting suspension was dispensed and introduced into a cryogenic vial and hold for 30 minutes in an ice bath. The cryogenic vials were introduced in to the –20° C freezer and under controlled freeze at 1^0 C min^{-1} in 45 minutes and to be kept for freezing for 30 days and six months [16].

Freezing protocol

The microalgae were preserved in liquid nitrogen using single step and two step freezing protocol as previously described by Day et al. [17].

Measurement of cell growth

The cell growth was analyzed using Neubauer counting chamber under the microscope and the total number of cells was calculated using the following formula:

$$\text{Total cell count} = \frac{\text{No of cells counted}}{\text{Total no.of squares counted}} \text{x Total no.of squares x } 10{,}000 \quad (1)$$

Thawing, recultivation and viability assay

Cryotubes were thawed by removing them from the cryogenic container and plunging them into a water bath for 5 min, at 35° C. The contents of the cryotubes were diluted by step-wise addition of 500 µl of their standard growth medium (Conway media). After equilibriation in the dark for 40min, the thawed microalgae were allowed to grow. 5 ml of standard growth medium added with 0.5 ml of 10^6 cells/ml^{-1} was treated as control. Control cultures of algae were processed without any treatment under the same conditions described above. The growth of post-thawed microalgae was assessed for 8 days against control, by counting individual cells using haemocytometer under microscope. Moreover, cells were examined for viability by microscopy. In order to compare the growth with control, the specific growth rate µ (day^{-1}) and cell yield were determined and compared with those of the control samples. The results were normalized to the control and expressed as relative values (%), giving an indirect measurement of viability as previously described by Tzovenis et al. [18]. The experiments were repeated three times with triplicate. µ was calculated using the formula.

$$\text{The specific growth rate } \mu = \text{Ln } (N_t / N_0) / t - t_0 \quad (2)$$

Where,

N_t = is the cell density at the end of exponential phase

N_o = is the cell density at the start (inoculums or cell density at the end of the lag phase)

t and to = are the corresponding times

Statistical Analysis

The data were subjected to statistical analysis using IBM SPSS software version no. 21 (SPSS Inc., Chicago, IL, USA). The statistical analyses were done by using one-way analysis of variance (ANOVA) followed by SNK tests. *P*-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Growth and viability of cryopreserved microalgae using single step freezing protocol in 30 minutes equilibration time and 30 days incubation

In this study, the microalgae, such as *Nannochloropsis salina*, *Chlorella volutis*, *Cheatoceros gracilis*, *Dunaliella* sp., and *Amphora* sp. were preserved with the above said cryoprotectants concentration in liquid nitrogen using single step freezing protocol showed no growth in the preserved culture after thawing. The *Nannochloropsis oculate* was unable to cryopreserve using the single step freezing protocol [19]. Consequently, the study recommended that the slow cooling protocol for the preservation of *Chlorella vulgaris*, *Dunaliella salina* and other green algae when preserved using liquid nitrogen. In rapid single step freezing protocol, the microalgae

samples in cryovials were directly plunged into liquid nitrogen (–196° C). Cells will be frozen rapidly in both extracellularly and intracellularly. The fatal intracellular ice formation should be prevented [20,21]. In this freezing technique there is no time for water loss from the cell to minimize intracellular ice formation, resulting in lower viability [22].

Growth and viability of cryopreserved microalgae *Nannochloropsis salina* using two step freezing protocol in 30 minutes equilibration time and 30 days incubation

The *Nannochloropsis salina* preserved with 10% DMSO showed the maximum viability of 91% survival with the maximum cell density of 207 ± 7.84 × 10^6 cells ml^{-1} followed by 15% methanol with a viability of 86% survival showed a maximum cell density of 184.67 ± 6.32 × 10^6 cells ml^{-1}, 2.5% glycerol having a viability of 72% survival with the maximum cell density of 132 ± 5.75 × 10^6 cells ml^{-1} and 12.5% ethylene glycol showed viability of 73% survival with the maximum cell density of 140.83 ± 6.33 × 10^6 cells ml^{-1}. The unfrozen control showed 100% survival with the maximum cell density of 245.67 ± 7.41 × 10^6 cells ml^{-1} (Figures 1 and 2). The one-way ANOVA showed that the viability significantly differed ($P<0.05$) among the different concentration of cryoprotectants. A study stated that the *Nannochloropsis oculata* was the most successful microalgae tested in his study, showing high tolerance to a wide range of cryopreservation conditions. The *Nannochloropsis oculata* presented good responses when treated with 5% DMSO, 10% DMSO, 5% methanol, and without any cryoprotectant [19]. This study confirms our result.

Growth and viability of cryopreserved microalgae *Chlorella volutis* using two step freezing protocol in 30 minutes equilibration time and 30 days incubation

In this study, the *Chlorella volutis* cryopreserved with 10% DMSO showed the maximum viability of 83% survival with the maximum cell density of 113.67 ± 5.25 × 10^6 cells ml^{-1} followed by 15% methanol with a viability of 94% survival with the maximum cell density of 117.33 ± 3.86 × 10^6 cells ml^{-1}, 2.5% glycerol having a viability of 62% survival with the maximum cell density of 111 ± 5.89 × 10^6 cells ml^{-1} and 15% ethylene glycol showed the viability of 52% survival with the maximum cell density of 77 ± 2.94 × 10^6 cells ml^{-1}. The unfrozen control had 100% survival with the maximum cell density of 125 ± 8.16 × 10^6 cells ml^{-1} (Figures 3 and 4). The one-way ANOVA showed that the viability significantly differed

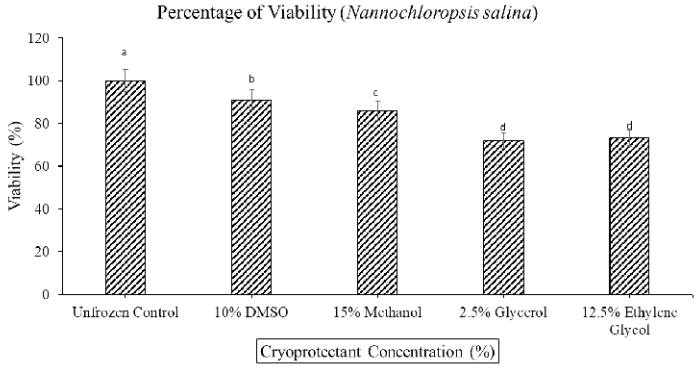

Figure 1: Viability percentage of *Nannochloropsis salina* preserved in 30 minutes equilibration time at -196°C for 30 days. Means with different lowercase letters are statistically different from each other.

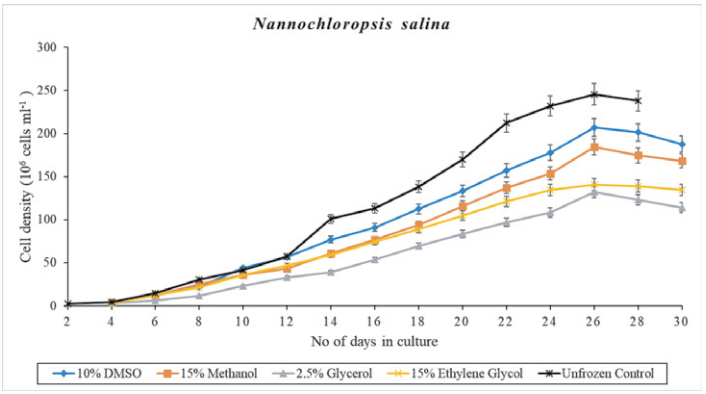

Figure 2: Graph showing the growth kinetics of *Nannochloropsis salina* preserved with different concentration of cryoprotectants in 30 minutes equilibration time.

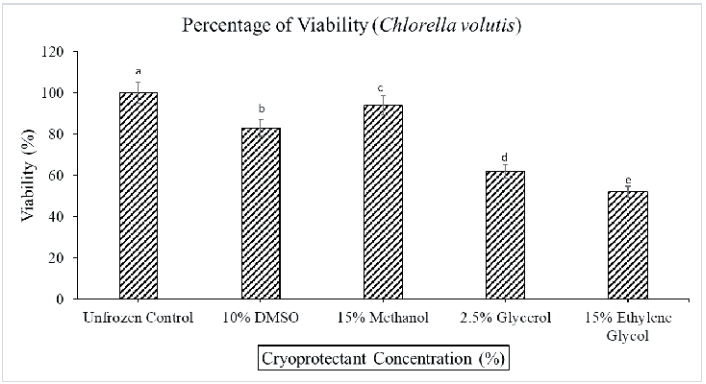

Figure 3: Viability percentage of *Chlorella volutis* preserved in 30 minutes equilibration time at -196°C for 30 days. Means with different lowercase letters are statistically different from each other.

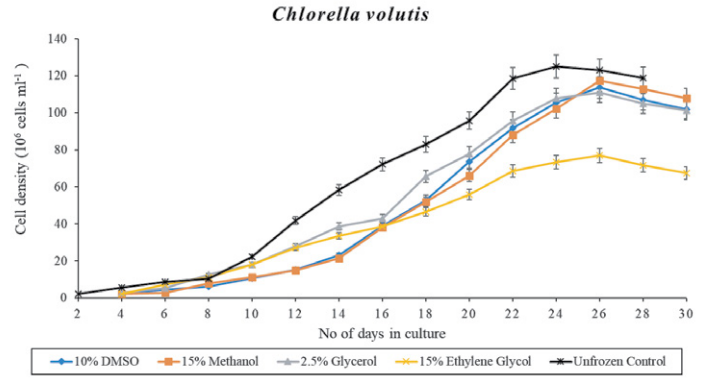

Figure 4: Graph showing the growth kinetics of *Chlorella volutis* preserved with different concentration of cryoprotectants in 30 minutes equilibration time.

($P<0.05$) among the different concentration of cryoprotectants. The *Chlorella vulgaris* reached 50% viability when cryopreserved with 10 and 15% DMSO [21] that was lower viability when compared with our findings. Slow cooling for *Chlorella vulgaris* and other green algae has been recommended when preserved using liquid nitrogen [20].

Growth and viability of cryopreserved microalgae *Cheatoceros gracilis* using two step freezing protocol in 30 minutes equilibration time and 30 days incubation

In this study, the *Cheatoceros gracilis* preserved with 10% DMSO showed maximum viability of 52% survival with the maximum cell

density of 59.67 ± 1.94 × 10^6 cells ml^{-1} followed by 10% methanol with a viability of 43% survival with the maximum cell density of 54.33 ± 1.04 × 10^6 cells ml^{-1}. The unfrozen control showed 100% survival with the maximum cell density of 126 ± 6.18 × 10^6 cells ml^{-1} (Figures 5 and 6). The one-way ANOVA showed that the viability significantly differed ($P<0.05$) among the different concentration of cryoprotectants. All hatchery feed diatoms, including *Chaetoceros muelleri*, *Chaetoceros calcitrans*, and *Chaetoceros* sp., and the larger benthic diatom *Nitzschia ovalis*, plus the two haptophytes, *Isochrysis galbana* and *Pavlova lutheri*, were successfully cryopreserved in 10 and 15% DMSO [23]. The maximum mean viability of *Cheatoceros gracilis* reached in this study was 52% but when compared to the previous study [24-26], the mean viability of *Cheatoceros gracilis* was below 40%.

Growth and viability of cryopreserved microalgae *Dunaliella* sp. using two step freezing protocol in 30 minutes equilibration time and 30 days incubation

In this study, the *Dunaliella* sp. preserved with 10% DMSO showed the maximum viability of 61% survival with the maximum cell density of 70.83 ± 2.49 × 10^6 cells ml^{-1}. The methanol, glycerol and ethylene glycol showed no viability in *Dunaliella* sp. The unfrozen control showed 100% survival with the maximum cell density of 85.67 ± 4.18 × 10^6 cells ml^{-1} (Figures 7 and 8). The one-way ANOVA showed that the viability significantly differed ($P<0.05$) among the different concentration of cryoprotectants. The 60 and 90% of viability in *Dunaliella tertiolecta* when cryopreserved – 80 °C resulting with the help of cryoprotection [17]. The *Dunaliella salina* showed the maximum viability of 72.7% and it was recorded in slow freezing protocol [20]. Moreover, the 15% DMSO seemed to be lethal for the *Dunaliella* cells.

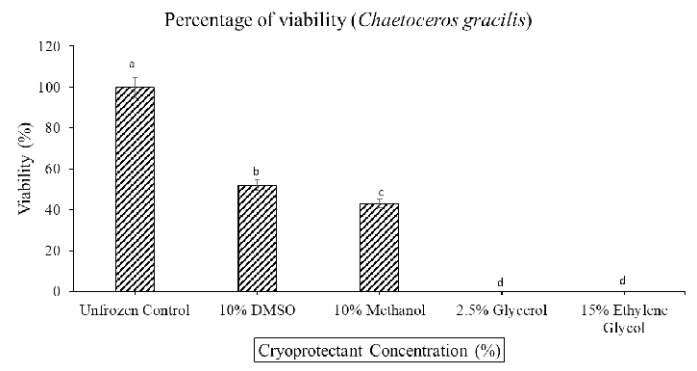

Figure 5: Viability percentage of *Chaetoceros gracilis* reserved in 30 minutes equilibration time at -196°C for 30 days. Means with different lowercase letters are statistically different from each other

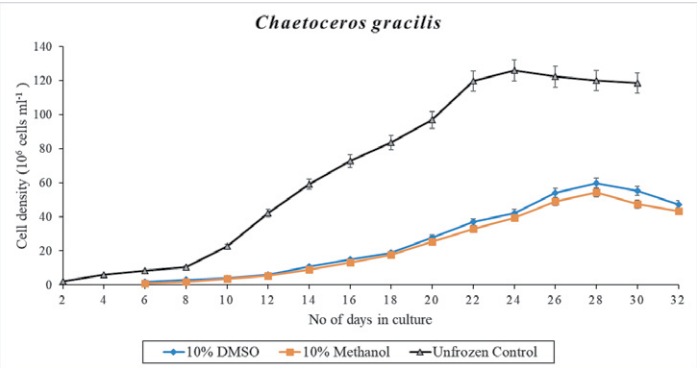

Figure 6: Graph showing the growth kinetics of *Chaetoceros gracilis* preserved with different concentration of cryoprotectants in 30 minutes equilibration time.

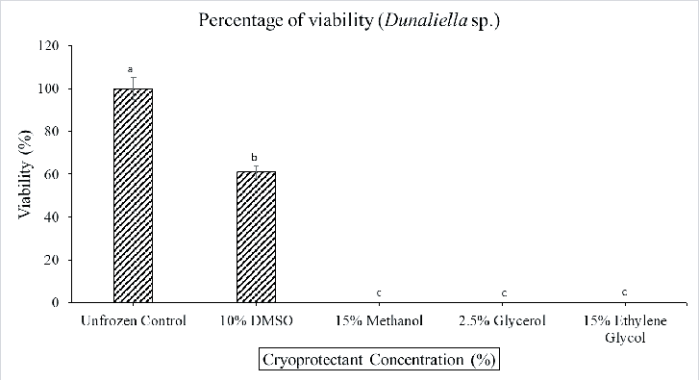

Figure 7: Viability percentage of *Dunaliella* sp. preserved in 30 minutes equilibration at -196°C for 30 days. Means with different lowercase letters are statistically different from each other.

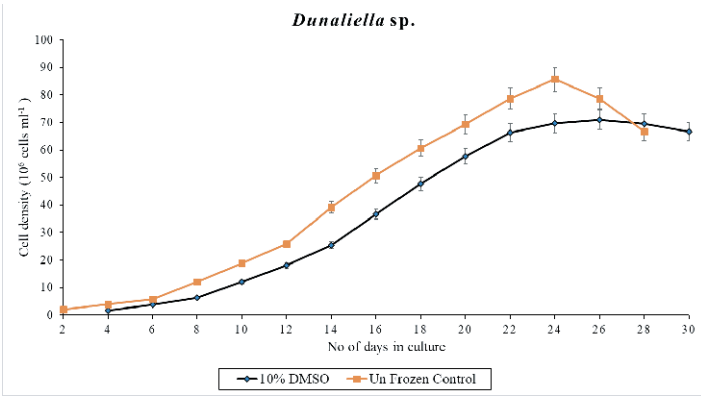

Figure 8: Graph showing the growth kinetics of *Dunaliella* sp. preserved with different concentration of cryoprotectants in 30 minutes equilibration time.

Growth and viability of cryopreserved microalgae *Amphora* sp. using two step freezing protocol in 30 minutes equilibration time and 30 days incubation

In this study, the *Amphora* sp. preserved with 10% DMSO showed the maximum viability of 69% with the maximum cell density of 21 ± 0.81 × 10^5 cells ml^{-1} followed by 15% methanol that had 66% of survival with the maximum cell density of 20.50 ± 0.81 × 10^5 cells ml^{-1}, 2.5% glycerol having a viability of 51% survival with the maximum cell density of 15.5 ± 1.41 × 10^5 cells ml^{-1} and 15% ethylene glycol showed the viability of 59% with the maximum cell density of 19 ± 1.41 × 10^5 cells ml^{-1}. The unfrozen control showed 100% survival with the maximum cell density of 28.83 ± 1.25 × 10^5 cells ml^{-1} (Figures 9 and 10). The one-way ANOVA showed that the viability significantly differed ($P<0.05$) among the different concentration of cryoprotectants. The cryopreserved diatoms, *Amphora cf. capitellata* showed highest viability when preserved with 10% DMSO for six months in the liquid nitrogen [21].

Viability percentage of cryopreserved microalgae *Nannochloropsis salina, Chlorella volutis, Cheatoceros gracilis, Dunaliella* sp. and *Amphora* sp. preserved at –196°c using two step freezing protocol in 30 minutes equilibration time after 6 months incubation

The successful cryoprotectants concentrations were taken from

each microalgae cryopreserved for 30 days to study the long term preservation of the microalgae.

In this study, the *Nannochloropsis salina, Cheatoceros gracilis, Dunaliella* sp. and *Amphora* sp. cryopreserved with 10% DMSO showed the maximum viability of 91, 48, 61 and 68% respectively. Likewise, the *Chlorella volutis* preserved with 15% methanol showed 93% survivability (Figure 11). The one-way ANOVA for 6 months cryopreservation showed that the viability significantly differed ($P<0.05$) among the different microalgae. Our study showed that the meager reduction in the viability of microalgae when preserved for 6 months than 30 days of cryopreservation.

Viability percentage of microalgae *Nannochloropsis salina, Chlorella volutis, Cheatoceros gracilis, Dunaliella* sp. and *Amphora* sp. preserved at – 20˚c using two step freezing protocol in 30 minutes equilibration time after 6 months incubation

In the present study, the microalgae *Nannochloropsis salina, Cheatoceros gracilis, Dunaliella* sp. and *Amphora* sp. were preserved with 10% DMSO and the *Chlorella volutis* was preserved with 15% methanol. All the microalgae were preserved at – 20˚C using the same protocol used for cryopreservation.

The microalgae *Nannochloropsis salina, Chlorella volutis, Dunaliella* sp. and *Amphora* sp. showed the maximum viability of 88, 91, 44 and 41% respectively and the microalgae *Cheatoceros gracilis* failed to regenerate when preserved at – 20˚C (Figure 12). This study

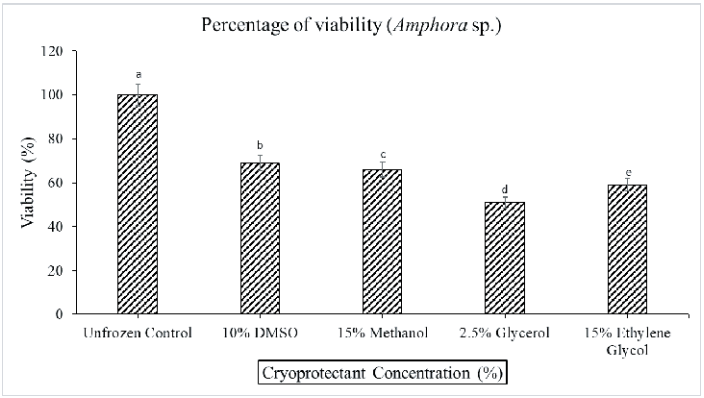

Figure 9: Viability percentage of *Amphora* sp. preserved in 30 minutes equilibration time at -196°C for 30 days. Means with different lowercase letters are statistically different from each other.

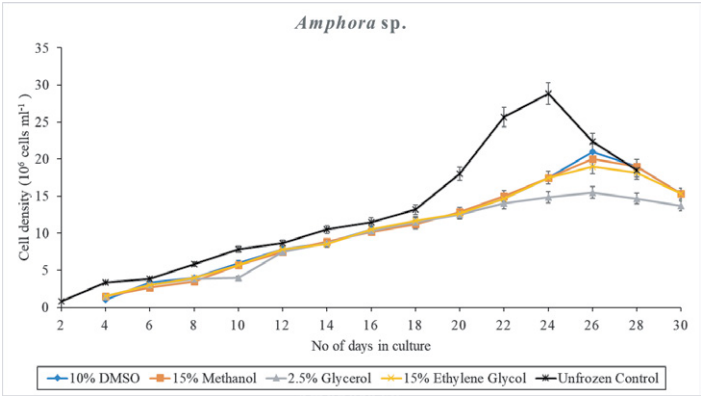

Figure 10: Graph showing the growth kinetics of *Amphora* sp. preserved with different concentration of cryoprotectants in 30 minutes equilibration time.

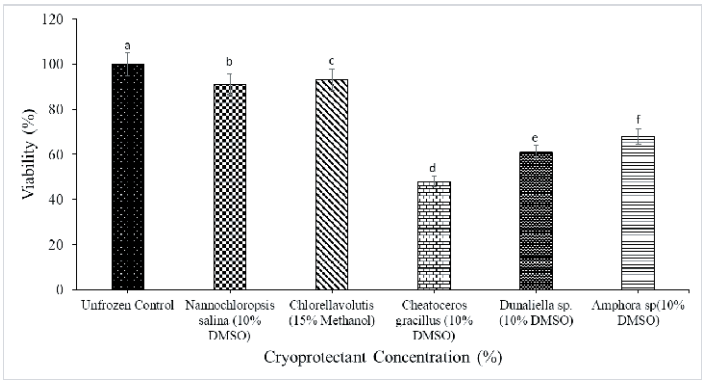

Figure 11: Viability percentage of different microalgae preserved in 30 minutes equilibration time at -196 ˚C for 6 months. Means with different lowercase letters are statistically different from each other.

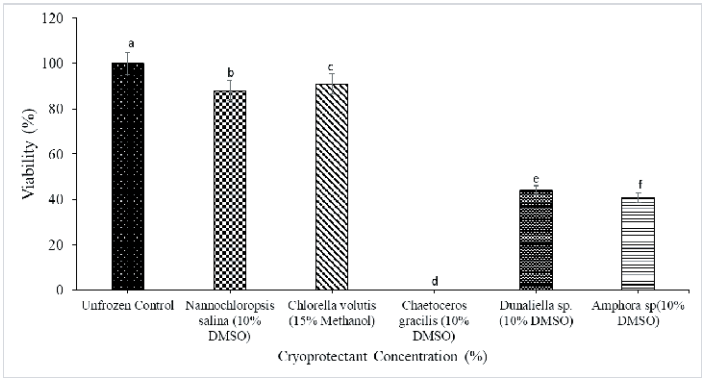

Figure 12: Viability percentage of different microalgae preserved in 30 minutes equilibration time at -20˚C for 6 months. Means with different lowercase letters are statistically different from each other.

showed that a negligible reduction in the viability of microalgae like *Nannochloropsis salina* and *Chlorella volutis* when preserved for 6 months at – 20˚C, but in the case of *Dunaliella* sp. and *Amphora* sp. that showed more variation in the mean viability at – 20˚C when compared to – 196˚C cryopreservation. The one-way ANOVA for 6 months cryopreservation showed that the viability significantly differed ($P<0.05$) among different microalgae. The *Chlorella minutissima, Chlorella stigmatophora, Chlorella capsulate, Dunaliella tertiolecta and Isochrysis galbana* exhibited good potential for cryopreservation at – 80˚C with and without cryoprotectants whereas at – 20˚C the results were less favorable [27]. From this study, it was confirmed that the preservation of microalgae can be possible at – 20˚C only with the help of cryoprotectants.

CONCLUSION

The study showed that the storage of algal stock cultures can be successfully preserved at – 20˚C and – 196˚C (cryopreservation). DMSO and methanol showed the maximum survival in all the preserved microalgae. A negligible reduction in the viability of microalgae were observed in the stock cryopreserved at – 20˚C and – 196˚C for 6 months incubation time. The uniqueness of the study is that the preservation of microalgal stock culture was possible at low temperature (– 20˚C) using cryoprotectants, but without liquid nitrogen. This technique is very cheap, affordable, less labor and avoiding the usage of liquid nitrogen.

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