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Induced Freezing and Desiccation Tolerance in the Microalgae Wild Type *Nannochloropsis* sp. and *Scenedesmus dimorphus*

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Abstract: The induction of desiccation and freezing tolerance in one marine and one fresh water unicellular algae strains was assayed by culture density and chlorophyll a content when treated cultures were grown in liquid medium. Both strains showed significant growth inhibition after desiccation. Acquisition of cell tolerance to slow desiccation to air dried state facilitated faster recovery growth when compared to fast desiccation. Investigated desiccated algae strains withstand one year freezing at -15^{0}C. Fresh water alga *Scenedesmus dimorphus* was more tolerant to desiccation and freezing than marine alga of *Nannochloropsis* sp. and slow-dried cells of frozen *Scenedesmus dimorphus* strain also displayed significant increase in cell density ($p<0.05$) and chlorophyll a content ($p<0.05$) compared to untreated cells. It is anticipated that further research and understanding on the basic mechanisms of induced desiccation and freezing tolerance and empirical development of improved protocols could expand the number and diversity of algal species that can be successfully preserved and stored at -15^{0}C without cryoprotectant.

Key words: *Nannochloropsis*, *Scenedesmus*, wild type, Induced freezing, desiccation tolerance,

INTRODUCTION

Algae can survive desiccation has been on record in the scientific literature for over a century. A useful list containing close to 420 species of desiccation tolerant algae, mainly greens and blue-greens, has been compiled by Davis (1972). Studies based on field and laboratory experiments have shown that some cyanobacteria (*Phormidium*, *Nostoc*) and some algae (*Prasiola*, *Zygnema*) are able to tolerate prolonged desiccation.

It has been demonstrated that the primary colonizers of the soils and water are bacteria, Cyanobacteria and algae. It may be hypothesized that the earlist colonizers are subjected to the greatest extremes of environmental stress. Therefore, it is these organisms which may possess the greatest resistance to environmental stress, and which may be used to test the hypothesis of environmental control of community development.

To clarify some of the terminology to be found in this paper the following comments is necessary. An algae which can survive desiccation is one which can revive from the air-dried state (the air being of low relative humidity) and an algae which is desiccated is one from which all available water has been lost to the surrounding dry atmosphere. Algae that can survive desiccation and can suspend its metabolism in the dry state will be termed desiccation tolerant, and which can survive freezing and can suspend its metabolism in the freezing state will be termed freezing tolerance (Bewley, 1979). The mechanisms of desiccation or freezing tolerance are not well understood despite the fact that numerous prokaryotic and eukaryotic organisms are capable of surviving more or less complete dehydration or extreme low temperature, and the current intense interest in long-term storage and survival of cells (Kennedy *et al*., 1994; Daniela Billi and Malcolm Potts, 2000). In general, water plays a crucial role for all metabolic activities and cellular dehydration can inhibit photosynthesis (Gray *et al*., 2007).

When cyanobacteria and algae from Antarctic wetland habitats tested for freezing and desiccation stress, desiccation was usually more injurious than freezing, especially for green algae that showed high mortality. More injurious was the exposure to a week of desiccation at 20^{0}C with an average viability of 14%. Dry tolerance was determined mostly by the ability to restore photosynthesis and respiration after rehydration (Sabacka and Elster, 2006). It has been suggested that the accumulation of fats and oils in the protoplasm of some algae (may be during water loss) increases their capacity to withstand desiccation (Evans, 1958; 1960).

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Despite the many algae species available for study, remarkably little work has been done to determine the physiological responses of algae to drying and rehydration.

Some algae survive slow desiccation better than rapid desiccation. Slow drying seems to enhance survival, perhaps by allowing cells to interact longer with environmental organic substances conferring some degree of protection (Holstetter and Hoshaw, 1970).

Many academic and industrial research laboratories maintain culture collections of living organisms which represent key resources with unique potential for research and development in diverse field of live sciences, including commercial development of biotechnology.

Cryopreservation is a method with significant potential for ensuring the long term conservation and genetic stability of micro algal cultures. A few freshwater and some marine species have been successfully cryopreserved but it is widely recognized that many factors potentially influence the success of cryopreservation, including the state and density of the culture, the nature and concentration of cryoprotectant, the composition and osmolarity of the medium, the rate of cooling and of thawing, and post thawing culture methods (Taylor and Fletcher, 1999). Low temperature acclimation at 4^0C for 4 weeks has induced freezing tolerance of unicellular marine algae in liquid nitrogen (Ben-Amotz and Gilboa, 1980). Testing the freezing and desiccation tolerance of algae has become important in terms of application to the cryopreservation of culture collection (Taylor and Fletcher, 1999).

Freeze-drying is a practical and inexpensive means of preserving microorganisms, which have been successfully applied in the areas of medical and industrial microbiology. There is evidence from a number of publications many alga species and Cyanobacteria have the ability to withstand desiccation and freezing tolerance. When freezing and desiccation tolerance of green alga species of *Klebsormidium* isolated from various habitats ranging from Antarctic to central Europe, where they occupied an environment where both seasonal and diurnal variations of water availability prevailed, they all found well adapted to freezing and desiccation injuries.

The aim of this study was to assess the induced desiccation and freezing tolerance in fresh water and marine unicellular algae. The study also aims to use such method for long-term storage and survival of algae strains. Chlorophyll *a* content and cell number growth measurements are used to monitor recovery from desiccation and freezing stress, and relate them to the rate of desiccation. Among the algal strains maintained at LARB (Laboratory for Algae Research and Biotechnology) the unicellular marine alga wild type *Nannochloropsis* sp. and fresh water alga *Scenedesmus dimorphus* have considerable potential biotechnological exploitation including for biofuel production.

MATERIALS AND METHODS

Organisms and Culture Conditions:

Unicellular wild type algal strain *Nannochloropsis* sp. and fresh water alga *Scenedesmus dimorphus* were selected from LARB (Laboratory for Algae Research and Biotechnology, Arizona State University, USA) collection based on increased biomass production. These microalga are eukaryotic photosynthetic microorganisms that grow rapidly due to their simple structure. Cells of *Nannochloropsis* sp were cultured aseptically in sea water base (35g L^{-1} sea salt mix manufactured by Oceanic) on modified Guillard f2 medium (Guillard and Ryther, 1962) enriched with $NaNO_3$ (8.82 mM), NaH_2PO4 (.22 mM), four times the amount of trace metal mix, and vitamin mix reported in Guillard f/2 medium, and BG-11 (Rippka *et al*., 1979) for fresh water species *Scenedesmus dimorphus*. The cultures were grown in 250 ml vertical tubular columns (29mm ID and 60 cm height). Filtered compressed air/CO_2 mixture (99/1 v/v) was injected through a capillary tube at the bottom of the column for mixing, support growth and pH regulation for 6 d, until the end of log phase. The culture temperature was maintained at 25^0C illuminated continuous at light integrity of 80 mol photons $m^{-2}s^{-1}$ on the surface of the outer tube with cool light fluorescent bulbs and pH of the experiments was maintained at 7.8±0.3 throughout. The culture for both strains was grown until late exponential growth stage and the cells were subjected to slow or fast desiccation (drying) on the seventy days.

Drying and Freezing Procedure:

For drying, the cell culture of wild type *Nannochloropsis* sp. and *Scenedesmos dimorphus* were centrifuged (3000 rpm for 10 min) to obtain concentrated cell sediments. The cells were passed through atmospheres of progressively reduced relative humidity with intermittent air drying at the latter stage arriving at the air dry situation on the third day for fast drying and sixth day for slow drying until constant weight had been attained. The relative humidity was generated in desiccators containing saturated solutions with varying humidity level

(Anandarajah and McKersie, 1990). Cell weight or both strains were determined daily for calculations of water loss until it reached air dry conditions (triplicates). The cells of slow and fast-dried samples were kept in air-dried state for 7 days. Half of the air-dried cell samples were frozen at -15^{0}C after 4 days of acclimation, 2 days at 4^{0}C,and 2 days at -4^{0}C. The frozen samples were maintained -15^{0}C for one full year. Other halves of the samples were used to study the effect of desiccation on algal cell growth.

Preparation for Culture Growth Studies:

After storage period of 7 days, air-dried samples were slowly hydrated with high relative humidity generated in a desiccators containing water as to prevent cells from experiencing excessive osmotic pressure when they are exposed to liquid medium from dry state. After freeze-storage period of one year, the frozen cells (previously desiccated and rehydrated) of both strains were taken from the freezer. After 24 hr equilibration at +4^{0}C and 24 hr equilibration at room temperature (25^{0}C), initial cell density of both strains was adjusted. To determine the effect of desiccation on cells growth initial cell density *Nannochloropsis* sp and *Scenedesmus dimorphus* were adjusted to 20×10^6 cells ml^{-1} and 5×10^6 cells ml$^-$ respectively, the initial cell density for both strains were doubled the concentration of cells used for desiccation studies. Cultures were maintained for 12 days for each experiment underroom conditions described above.

Growth Assessment:

Cell growth after desiccation was determined by the ability of cells to actively divide and multiply when compared with undried control cells. We have adopted the turbidity approach to assessing growth of both microalgal strains. Turbidity is determined through absorbance readings at 750 nm on a standard spectrophotometer. The relationship between turbidity and cell counts was established through the generation of a standard curve comparing manual microscopic cell counts using a hemacytometer under alight microscope and absorbance based on three subsamples. R^2 for the relationship between absorbance and cell counts is 0.990 for *Nannochloropsis* sp and .0.969 for *Senedesmus dimorphus* illustrating the potential for a high degree of confidence in predicting cell counts from green algal culture absorbance readings over the range of the curve.

Chlorophyll a Content:

Microalgal cells were extracted in methanol and the chlorophyll *a* content was determined spectrophotometrically by measuring the optical density of methanol extract against a methanol blank at 665 and 750 nm (Azov, 1982) with a spectromax 340 PC (Molecular Device) spectrophotometer. The chlorophyll *a* concentration was determined by using the coefficient given by Talling (1969) in the following equation; Chlorophyll a (mg per liter) = 13.9 (O.D.750).U.V; in which O.D. = Optical density, U=the final methanol volume, and V= the sample volume.

Statistical Analysis:

All experiments were carried out in triplicate, and all data represent the either the mean and statistical significance or mean and standard deviation of three replicate measurements. Cell growths that compare performance of desiccated and undesiccated cells were analyzed by paired *t*-test using Graph Pad software.

RESULTS AND DISCUSSION

Our result exhibited that (Fig: 1). shows the induced drying regimes and the rate of fresh weight loss for cells of wild type *Nannochloropsis* sp. on daily basis until they reached air dry situation (approximately 32.1% wt/wt). The air dried cells contained approximately 6.6% water when compared to oven dry weight. The rate at which drying of cells occurs during desiccation process is critical for the ability of the desiccated cells acquire a tolerance of desiccation and to restore cell growth. This is to be discussed further.

Effects of Desiccation on Algal Cells Growth:

The difference in aquatic habitat between the two algae is also demonstrated in their ability to recover after desiccation to air dry condition. Cell density calculated from turbidity measurement after 6 days revealed that each desiccation treatment induced a significant decrease in cell density compared to undried control culture (Table 1) for wild type *Nannochloropsis* sp and *Scenedesmus dimorphus.* However slow or fast drying of cells retained their ability to recover the cellular and metabolic activity. Cell density in slow and fast drying treatments increased from the 6th day, and subsequently, the discrepancy between the control and desiccation treatment narrowed down. On 12th day, the mean cell density of slow drying treated cultures of

Nannochloropsis sp. and *Scenedesmus dimorphus* achieved 84.6% and 96.5% of non dried controls, respectively. The mean cell density of slow- dry regime cultures of *Scenedesmus* not significantly different from control ($13.9x10^{-6}$ and $14.4x10^{-6}$, respectively). However, cell density of rapid drying treated cultures of *Nannochloropsis* sp. and *Scenedesmus dimorphus* ($25.8x10^{-6}$ and $11.4x10^{-6}$, respectively) significantly decreased from respective undried controls ($p<0.01$). Despite an initial decrease in cell division, both strains were not prevented from dividing and cultures of both strains rapidly achieved optimal densities, though it was more pronounced in cultures of *Scenedesmus dimorphus* when compared that with cultures of *Nannochloropsis* sp.

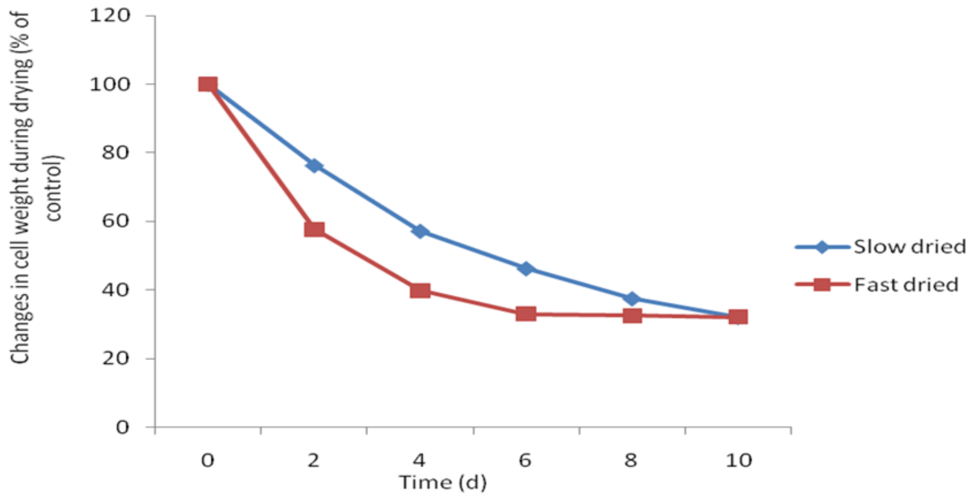


Fig. 1: Loss of cell water during slow and fast drying of *Nannochloropsis* sp.until it reached air dry weight.

Table 1: Effect of drying regimes on the cell growth of wild type *Nannochloropsis* sp. and *Scenedesmus dimorphus*. Each data point represents net cell growth over initial cell density. Data are the means of triplicate cultures.

Drying regimes	Cell density ($X10^{-6}$)	Relative growth (% of control)	Cell density ($X10^{-6}$)	Relative growth (% of control)
Nannochloropsis sp.	6d	6 d	12d	12d
undried(cont.)	14.3	100.0	45.7	100.0
slow dried	9.5*	70.0	36.2*	84.6
fast dried	4.9**	55.4	22.4**	64.5
Scenedesmus dimorphus	5.1	100.0	13.8	100.0
undried(cont.)				
slow dried	3.8*	76.1	12.9ns	96.5
fast dried	1.8**	38.0	10.8**	82.0

Difference between undried control and dried;
** Significance at P<0.01
* Significance at P<0.05
NS not significant

Effects of Desiccation and Freezing on Algal Cells Growth and Chlorophyll a Content:

The effect of drying regimes fottowed by one year freezing stress in *Nannochloropsis* sp. and *Scenedesmus dimorphus*, are show in Fig. 2a and Fig. 2b. Both strains, did withstand long term freezing with no cryoprotective treatment to prevent cells from potential freezing injuries. The combined effect of long term freezing of both strain displayed different recovery rate depending on the rate of drying. However, cultures frozen for one year after slow and rapid drying regimes retained their ability to divide in treated cultures when thawed samples were cultured in liquid medium.

Each desiccation regime followed by freezing treatment induced significant decrease ($p<0.01$) in cell density of *Nannochloropsis* sp. compared to control cultures when turbidity measurement was taken 2 d after thawed samples were cultured in the fresh enriched f/2 medium. The mean 2 d old culture densities of slow and rapid-dry treatments were 44 ± 3.10^{-6} $ml^{-1}$and 34 ± 2.10^{-6}, respectively (73.4±5% and 56.7±3% of control, respectively) of un dried unfrozen cell cultures. Subsequently the gap between the control and treated cultures progressively reduced. After 12 d, the mean cell culture densities of slow and rapid dried treatments were 82.6±3% and 69.7±3% of control, respectively). During 12 d period, untreated cultures of *Nannochloropsis* sp. displayed approximately eight fold increase in cell density from initial cell density of 40×10^{-6} ml^{-1}.

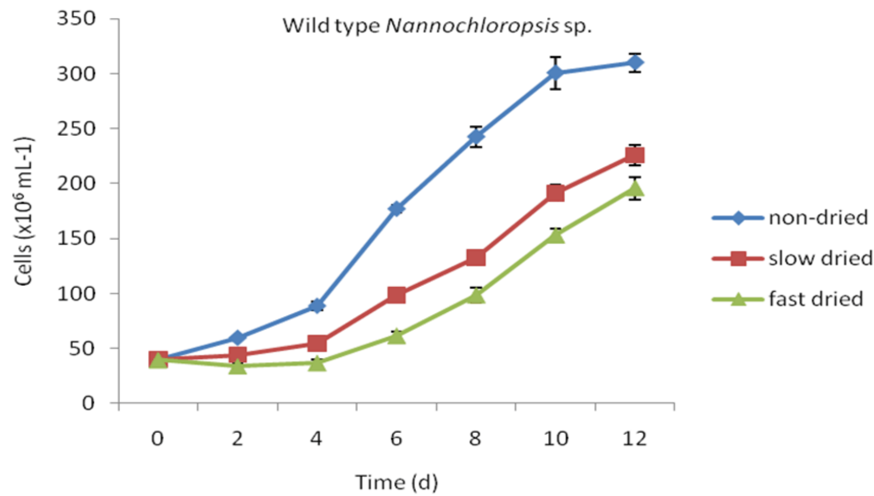


Fig. 2a: Growth curves of wild type *Nannochloropsis* sp. cultivatedin enriched f/2 medium after slow and fast desiccation of cells followed by freezing stress.

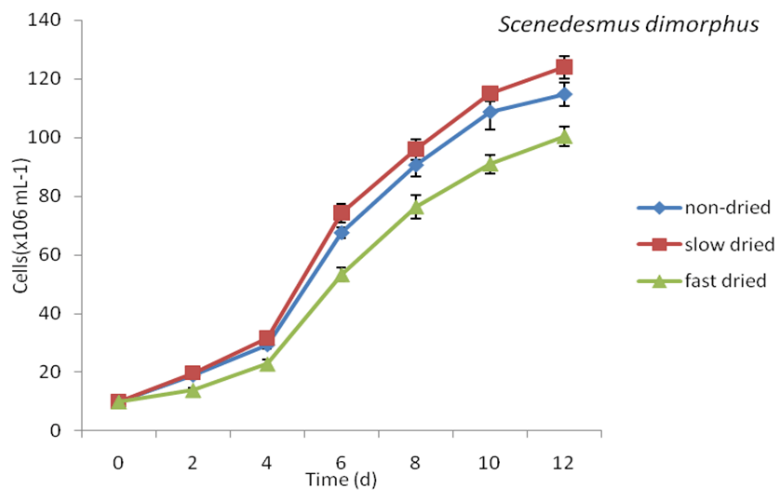


Fig. 2b: Growth curves of *scenedesmus dimorphus* cultivated in BG 11 medium after slow and fast desiccation of cells followed by freezing stress

Scenedesmus dimorphus displayed different post-thaw recovery rates depending on the drying regime employed prior to freezing. The cell density of frozen cultures subjected to slow drying prior to freezing remained same as control over the first 4 days. However, rapid drying treatment resulted in significant decrease ($p<0.05$) in cell density throughout the growth period compared to undried controls. After 6 d, the mean slow-dry treated cell culture densities were higher than that of control throughout the growth period (maximum 109.6±3.2%, $p<0.05$ on 12 d). The cell density of slow-dry treated thawed cultures increased from the 2nd to 12th d ($20\pm1.10^{-,6}$ ml^{-1} and $122\pm4.10^{-,6}$ ml^{-1}, respectively) by which time it exceeded the control value ($115\pm5.10^{-,6}$ ml^{-1}, $p< 0.05$).

Effects of Desiccation and Freezing on Algal Cells Chlorophyll a Content:

Chlorophyll a content was measured for both strains after one year exposure to freezing at -15^{0}C followed by 12 day post thaw culture in liquid medium (Fig.3). The strains *Nannochloropsis* sp. and *Scenedesmus dimorphus* displayed different post thaw recovery rates of chlorophyll a content depending on the drying regime employed prior to freezing (Fig. 3 a and b).

The two drying regimes employed prior to freezing resulted in significant chlorophyll *a* reduction in

thawed cultures of *Nannochloropsis* sp. on the 3rd day ($p<0.01$) (Fig. 3a). The reduction was approximately threefold and sevenfold lowers in the slow-dried and rapid-dried cells, respectively, than that of undried control which has resulted in a delay in the initiation of the normal growth phase. However in both regimes chlorophyll a values increased from the 3rd day, approaching control values on the 12th d (27.7±1.5 mg/L or 82.7±4.4% for slow-dried thawed cells and 21.7±1.5 mg/L or 64.9% for rapid-dried cells). Despite an initial severe decrease in chlorophyll a content, *Nannochloropsis* sp. cultures rapidly achieved optimal chlorophyll a content.

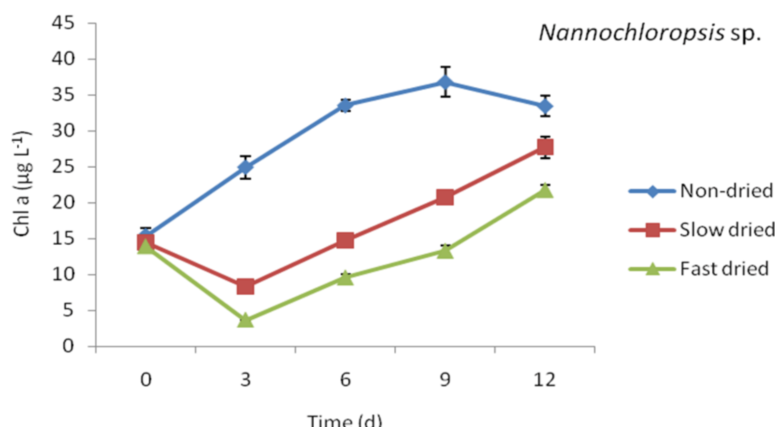


Fig. 3a: Chl *a* content of wild type *Nannochloropsis* sp. cultivated in enriched f/2 medium after slow and fast desiccation of cells followed by freezing stress

By contrast, slow-dried treatment followed by freezing resulted in a very efficient chlorophyll a synthesis in *Scenedesmus dimorphus* (Fig. 3b). The mean chlorophyll a content of thawed cultures progressively increased from the 3rd day to 12th d (42.6±2.0 mg/L and 64.8±2.0 mg/L, respectively) by which time it exceeded the undried unfrozen control value (53.6±1.9 mg/L). After 3rd day, mean chlorophyll *a* value of slow-dried treated cultures significantly exceeded that of the untreated control until 12th d ($p<0.05$). After 3 d, the mean chlorophyll a content of rapid-dried cultures decreased that of the control (28.3±1.4 and 37.7±1.8, $p<0.05$, respectively), and subsequently the discrepancy between the control and rapid-dry treated cultures progressively decreased. After 12 d, the mean chlorophyll a values of rapid-dried cultures were almost same as that of the control (53.6±2.3 mg/L and 53.8±1.9 mg/L).

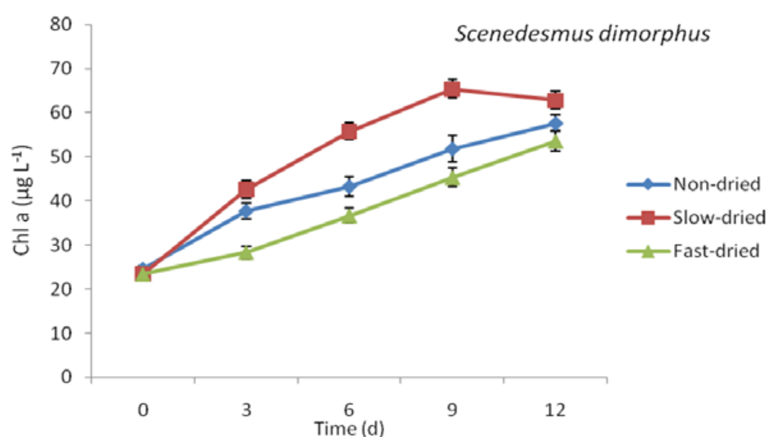
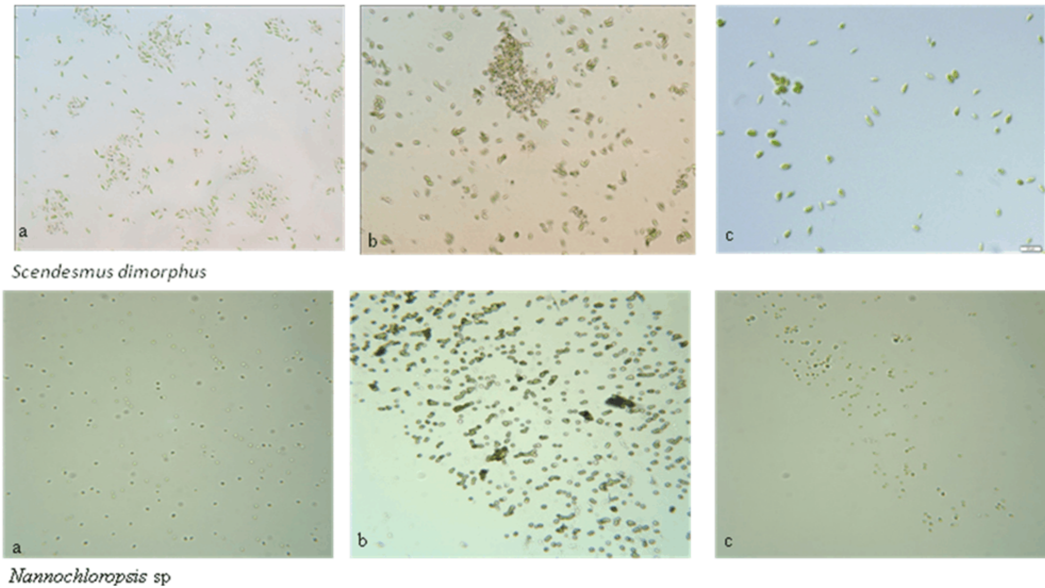


Fig. 3b: Chl *a* content of *scenedesmus dimorphus* cultivated in BG 11 medium after slow and fast desiccation of cells followed by freezing stress

After 12 d, the mean culture growth of *Nannochloropsis* sp. after exposure to slow and rapid desiccation compared to control cultures did not differ from yearlong freezing treatment after the both desiccation regimes (Fig.4). However, combined effect of desiccation and freezing significantly increased cell density and chlorophyll a content compared to culture.



A. First stage of strains, B. Aggregated condition, C. After freeze dry condition

Fig. 4: Effect of drying regimes on the cell growth of *Scenedesmus dimorphus* and wild type *Nannochloropsis* sp.

Though both strains reacted somewhat differently to desiccation and freezing, they have a similar response to both desiccation and freezing stresses and they were particularly resistant to desiccation and long-term freezing injuries. As for vascular plants, eukaryotic microalgae species in this case *Nannochloropsis* sp., *Scenedesmus dimorphus*, and also genus *Klebsomidium*, (Elster *et al*., 2008) are characterized by a certain genetically fixed level of resistance to low temperatures and desiccation. The level of resistance or tolerance varies among individual strains and/or species, and depends on the organism's origin and phylogeny. Both cold stress (chilling and freezing) and desiccation stress strongly influence metabolic process in the cells (Nishida and Murata, 1996; Potts 1999; Alpert, 2006). References have been made to the accumulation of granular bodies or oil globules in organisms exposed to periods of desiccation (Evans, 1958). Microalgae are suspected of synthesizing and storaging oily-type lipids and environmental stress factors have been studied with relation to light intensity, temperature, pH, nitrate and various other nutrient deprivations (Yu Suen *et al*., 1987; Hu and Sommerfeld, 2008). It is worth investigating the effect of partial desiccation or low temperature exposure as stress factors on synthesizing and storaging lipids.

Suitable desiccation treatment followed by carefully regulated cooling are both necessary to ensure recovery of such cells from -15^{0}C freezing. Of the two drying regimes tested, slow drying was the most efficient at protecting both strains under the experimental conditions described above. Four days of slow cooling rate and acclimation proved suitable as thawed cells were able to rapidly recover their ability to divide. Of the two drying regimes tested, slow-drying was more effective at protecting both marine and fresh water strains form freezing injuries under the experimental conditions described above.

Desiccation and long term freezing of cells of both microalgal strains selected for our studies had no permanent deleterious effects on subsequent growth. In fact, post culture growth of slow desiccation followed by one year long freezing actually increased the chlorophyll *a* content compared with control culture. Moreover, chlorophyll *a* accumulates faster in *Scenedesmus dimorphus* during one year storage period, and it also promoted cell growth compared to unfrozen control culture. The effects of the status of water content of cells on freezing tolerance are complex. It is possible that freezing took place extracellurlarly in both strains as the water content of the cells was low. It has been suggested that extracellular freezing and desiccation ultimately stress cells in a similar manner, namely that both lead to an increase in the osmotic stress on the cell through the deprivation of free water. Intracellular freezing is more likely to cause cellular damage to plant cells (Burke *et al.,* 1976). Apparently the effect of freezing stress on desiccated cells of microalgae does not lead to cellular damage.

The kinetics of recovery of carbon fixation after drying marine or fresh water algae do not appear to have been studied. Nevertheless, it is evident that the ability to tolerate desiccation and freezing and to resume photosynthesis and growth when exposed to aquatic environment is possible in species adapted to marine and fresh water habitats.

It is generally accepted that the extent to which any organisms such as algae and lichens can tolerate being dried out is related to the moisture conditions to which they are adapted in their natural habitat. Compared to aquatic habitats, algae colonizing aero terrestrial habitats are much more exposed to harsh and rapidly changing environmental conditions such as radiation, temperature, nutrient concentrations, and in particular desiccation stress (Karsten *et al*., 2007). Thus, it is anticipated that further research on the basic mechanisms of induced desiccation and freezing tolerance and empirical development of improved protocols will continue to expand the number and diversity of algal species that can be successfully preserved and stored in conventional freezers on a medium or long term basis. The method described here can be adapted to a wide range of algae without extensive training or expensive equipment. Thawing and reviving the frozen cultures are also simpler and less susceptible to further damage. It is a relatively inexpensive method and is beneficial for culture collections and the aquaculture industry.

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