Agronomy Research **14**(4), 1266–1273, 2016

Diatom cultivation and lipid productivity for non-cryopreserved and cryopreserved cells

Z. Demirel*, C. Demirkaya, E. Imamoglu and M. Conk Dalay

Ege University, Faculty of Engineering, Department of Bioengineering, TR 35100 Bornova-Izmir, Turkey
*Correspondence: zelihademirel@gmail.com

Abstract. Many freshwater and marine algae can be cryopreserved, but typically with lower post-thaw viability levels. However, most of the algae groups (dinoflagellates, cryptophytes, synurophytes, and raphidophytes) cannot be successfully cryopreserved in these days. Marine diatoms can be cryopreserved and frequently have shown great viability. The aim of this study is to compare the cultivation and lipid productivity for non-cryopreserved and cryopreserved marine diatom cells. Diatoms preserved in the EGEMACC (Ege University Microalgae Culture Collection) are usually maintained by serial sub-culturing. In this study, the cryopreservation of marine diatom algae (*Amphora cf. capitellata*, *Cylindrotheca closterium*, *Nanofrustulum shiloi*) using the passive freezing system procedure was studied. Investigation into the cause of the freezing injury at the cellular level was made at different salt concentrations. Passive freezing method used in sea salts liquid media at the percentage of 1%, 2% and 3% containing cryoprotectant of 10% Me_2SO for six months in liquid nitrogen. *C. closterium* was obtained with the highest viability however *N. shiloi* was revival extended period of time. All of the diatom cells were grown in 1 L sterile bottle containing 900 mL of F/2 medium under the light intensity of 20 μmol photons m^{-2} s^{-1} at 22 ± 2 °C with the air flow rate of 1 L min^{-1} for 15 days. The growth rate and biomass productivity were determined at the end of the batch production process. Also, lipid content of *A. capitellata* was obtained at the highest concentration compared to that of the other diatoms.

Key words: *Cylindrotheca closterium*, *Amphora* cf. *capitellata*, *Nanofrustulum shiloi*, cryopreservation, growth rate, lipid content.

INTRODUCTION

Microalgal biotechnologies are quickly developed for commercial exploitation of a range of products including health supplements, animal feeds, biofuels and chemical feed stocks (Bui et al., 2013). Diatoms have significant biotechnological potential as many taxa produce high levels of lipids, including high levels of triacylglycerols, making them candidates for aquaculture feeds, functional foods and even as a potential feedstock for biodiesel (Levitan et al., 2014; d'Ippolito et al., 2015).

Microalgae preserved in the Ege University Culture Collection (EGEMACC) 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 do not survive periodic, routine transfer of subcultures (Day et al, 1997; Tanniou et al., 2012; Piasecki et al., 2009).

Cryopreservation, with its potential for long-term conservation of biological resources, provides an invaluable tool to ensure the biosecurity and genotypic stability of model diatom taxa. *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* have been successfully cryopreserved using methodologies as two-step freezing protocols (Day & Brand, 2005). The preservation of organisms without change in their morphological, physiological, biochemical and genetic properties is a first function of culture collections. Moreover, a second function is a significant amount of research on the fundamental cause of cryopreservation induced injuries such as chilling injury, injury at subfreezing temperatures, extracellular ice formulation, intracellular ice formulation and osmatic stress in algae (Mori et al., 2002; Brand & Diller, 2004; Hedoin et al., 2006).

The optimum conditions to use during freezing and thawing with algae have developed experimentally. Some basic procedures have been used to cope with or minimize the many undesirable effects of freezing and thawing: (a) Organisms should be transferred to fresh growth media; (b) cryoprotectants in concentrations of 5 to 15% should be added to growth media; (c) time must be allowed for osmotic equilibrium between the cells and the cryoprotective medium; (d) the freezing rate must be controlled. Successful procedures avoid all changes in membrane permeability, gas solubility and salt concentrations within the cell (Saks, 1978; Taylor& Fletcher, 1999).

Microalgal cell is prepared before freezing where a cryoprotective agent (CPA) can be added to the cell suspension to avoid cellular damage due to ice formation. Two forms of artificial cryoprotectants may be distinguished, the penetrating (passively move through the plasma membrane to equilibrate between the extracellular solution and the cell interior) and the non-penetrating types (do not pass through the plasma membrane and remain in the extracellular solution). The second category of non-penetrating CPAs, such as polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG), less toxic for the cell, are scarcely used in cryopreservation (Fuller, 2004; Tanniou et al., 2012).

The maintenance of subculture algae collections based on agar, though well-established, is labour intensive, costly and subject to contamination and genetic change (Bui et al., 2013). Although studies are available on cryopreservation effect on marine algae, work on marine diatoms is inadequate (Redekar & Wagh, 2000).

The aim of this study was to investigate the potential of using cryopreservation to conserve marine diatoms, comparing the cultivation and lipid productivity of non-cryopreserved and cryopreserved applied to diatom cells.

MATERIAL AND METHODS

Organism

The three local strains isolated from Aegean Sea, *Amphora cf. capitellata* (EGEMACC 2), *Cylindrotheca closterium* (EGEMACC 45), *Nanofrustulum shiloi* (EGEMACC 44), were obtained from Ege Microalgae Culture Collection (http://www.egemacc.com/).

Cryopreservation protocols

All of the diatom cultures were harvested in the latter part of the logarithmic growth phase after incubation for 14 days. Harvested cell were resuspended with 1 ml of F/2 medium and cell were counted with *Neubauer* hemocytometer, adjusted to 1 x 10^7 cells ml^{-1}, prepared three repeatedly (Day & Stacey, 2007).

Freezing and Thawing: Dimethyl sulfoxide (Me_2SO) was used as a cryoprotectant in the study. The cultures were centrifuged, the excess media was removed, then 10% Me_2SO solution was added to media containing 1%, 2% and 3% sea salt, respectively, held at -20 °C for 30 minutes, held at -80 °C for an overnight, and then plunged the culture into liquid nitrogen at 196 °C during six months.

Recovery procedure: Thawing was carried out by immersing the vials in a 40 °C water bath, In order to remove cryoprotectant, the thawed cell suspensions were centrifuged and supernatant was removed. After that cells were resuspended with 5 ml of F/2, and then incubated in the dark for 24 hours at 22 ± 2 °C. The cells were incubated under 20 μmol photons m^{-2} s^{-1} at 22 ± 2 °C for 1 week.

Viability assay: After one day thawing, cell viability measured by a staining protocol using fluorescein diacetate (FDA). 50 μL of FDA stain stock solution was added to 1 ml culture, incubated at room temperature for 5 min, and the cells were observed by blue-light fluorescence microscopy. Viable cells seemed fluoresce green (FDA positive) and nonviable cells appear red or colorless. The images of living cells were taken under 485/535 nm with fluoresce in microscope (Olympus BX53, Japan) at 60X magnification. Viability was expressed as a percentage of control (nontreated unfrozen culture) vs FDA positive cells (Day & DeVille 1995).

Cultures

The thawed and non-cryopreserved strains were cultured in 1 L sterile bottle containing 900 ml of F/2 (Guillard and Ryther 1962, Guillard 1975), at 22 ± 2 °C, under white led lamps at an intensity of 20 μmol photons m^{-2} s^{-1} with the air flow rate of 1 L min^{-1} for 15 days.

Analytical Procedures

Cell growth (cells ml^{-1}) was estimated daily using a *Neubauer* counting chamber under an inverted microscope (Olympus CH40, Japan). The optical density of the culture was evaluated by using a spectrophotometer (Ultrospec 1100 pro, Amersham Bioscience), at λ = 600 nm. The specific growth rate (μ) of the cells was calculated from the exponential (straight line) phase, as $\mu = (ln N_1 - ln N_1)/d(t_2-t_1)$, where N_2 is the final cell concentration, N_1 is the initial cell concentration and dt is the time required for the increase in concentration from N_1 to N_2. Doubling time (DT) was also calculated as $DT = ln2\ \mu^{-1}$, according to Wood et al., 2005.

Morphological characterization of the strain was performed by light and florescence microscopy (Olympus BX53, Japan).

Diatoms were harvested by centrifugation (Pro-Research by Centrurion Scientific Ltd) at 5,000 rpm for 5 min. Cells were washed twice with distilled water to remove sea salt. The pellets were then lyophilized with a Christ (Alpha 1–2 LD plus, Germany) freeze dryer, to estimate the cell dry weight. The dried biomass obtained after freeze-drying was stored in air-tight containers at -20 °C. Biomass productivity was expressed as dry weight from 1 L of culture per days of algal growth.

Lipid was extracted from lyophilized diatom biomass using extraction solvent mixture of methanol/chloroform, described by Demirel et al. 2015.

The data were analyzed using one-way analysis of variance (ANOVA). A probability value of $p \leq 0.05$ was considered to denote a statistically significant difference, and $p \leq 0.01$ was also used to show the power of the significance. Results were reported as mean values with standard deviations ($n = 3$) unless otherwise indicated.

RESULTS AND DISCUSSION

Me_2SO was introduced into cryobiology as a very effective, rapidly penetrating, and universal the cryoprotective additive (Hubalek, 2003). 10% Me_2SO were useful cryoprotectant and 1%, 2% and 3% salt concentrations were the better medium in the majority of the combinations tested. The staining method of FDA is rapid and convenient for viability assay, as well as commonly used for microalgae (Mori et al., 2002). In this study, diatom cells appeared in six months after subculturing of the thawed strains. The dead cells had surface damage or ruptured membranes. The results showed that among the salt concentrations of cell viability was found the highest in 1% salinity. In other words, the cell viability was increased with decreasing the salinity concentration for the cryopreservation of marine diatoms. Osmotic alters in cells throughout ice formation was a primary factor negatively affecting viability (Canavate & Lubian, 1994). The effect of reducing the salinity of the external medium on diatom cells viability is shown in the data in Fig. 1.

In generally, penetrating cryoprotectant was used for marine microalgae such as methanol (MeOH), dimethylsulfoxide (Me_2SO) and glycerol (Gly). The three most used CPAs for the viability observed in frozen cells with dimethyl sulfoxide originates during the freezing period and the function of cryoprotectants is related to a decrease in risk of physical and chemical damage during the ice formation process (Grima et al., 1994).

Though Canavate & Lubian (1994) and Joseph et al. (2000) studied tolerance of *Chaetoceros gracilis* and *C. calciltrans* to Me_2SO, Methanol and showed that it completely lost viability when exposed to 20% concentration of Me_2SO was so much lethal. But safer concentrations for Me_2SO were at 5% and lower. Methanol was described as an affectless CPA for diatoms. Some marine microalgae are more effectively cryopreserved with Me_2SO compared to MeOH, whereas glycerol is effective for members of the genus *Tetraselmis* (Reed, 2007).

There are many reports for the growth rate of cultivated marine phytoplankton species isolated from coastal and oceanic habits (Scholz & Liebezeit 2012; Scholz, 2014). Non-cryopreserved and cryopreserved marine diatom species were grown in standard F/2 medium (Table 1).

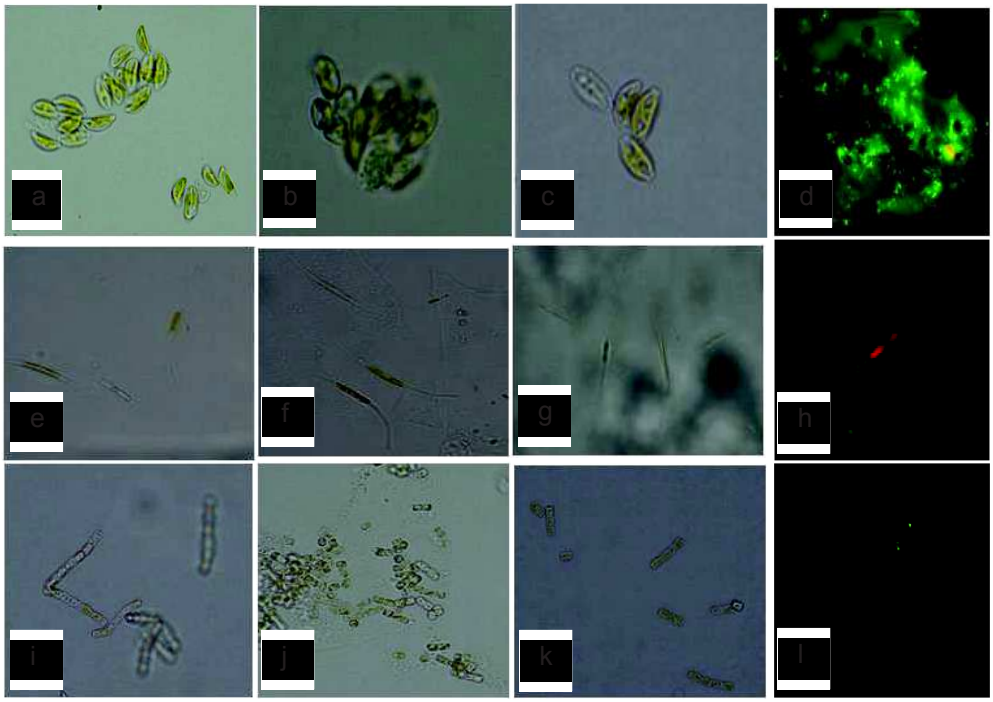

Figure 1. Diatom cells treated with 10% Me_2SO cryoprotectant. Figs a, b, c − *A. capitellata* with 1%, 2%, 3% salt concentrations, respectively, d − staining with 1% FDA 10% Me_2SO cryoprotectant; Figs e, f, g, *C. closterium* with 1%, 2%, 3% salt concentrations, respectively, h − staining with 1% FDA 10% Me_2SO cryoprotectant; Figs i, j, k, *N. shiloi* with 1%, 2%, 3% salt concentrations, respectively, l − staining with 1% FDA 10% Me_2SO cryoprotectant using light and florescence microscopy (63X magnification).

Table 1. Non-cryopreserved and cryopreserved diatom species were grown in F/2 medium

	Specific growth rate (μ; day^{-1})	Doubling time (day)	Dry weight (mg L^{-1})	Biomass Productivity (mg $L^{-1}day^{-1}$)	Percentage of lipids (%)
Amphora cf. capitellata	0.1072 ± 0.005	6.466	124.5	8.300 ± 0.001	**33.719 ± 0.244**
Amphora cf. capitellata-cryopreserved	0.2015 ± 0.015	3.441	212.8	14.187 ± 0.001	15.576 ± 0.208
Cylindrotheca closterium	0.1800 ± 0.012	3.851	312.8	20.853 ± 0.001	18.771 ± 0.083
Cylindrotheca closterium-cryopreserved	0.1750 ± 0.007	3.961	326.8	21.786 ± 0.003	25.150 ± 0.175
Nanofrustulum shiloi	0.2957 ± 0.018	**2.345**	366	**24.400 ± 0.001**	24.295 ± 0.163
Nanofrustulum shiloi-cryopreserved	0.2647 ± 0.023	2.619	259.1	17.273 ± 0.001	23.641 ± 0.346

Each diatom strains showed specific slope during the cultivation period (Fig. 2).

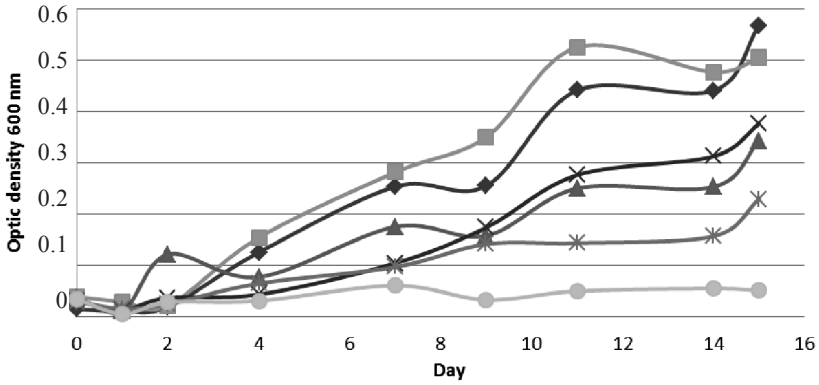

Figure 2. Growth profiles of cryopreserved diatom species. *C. closterium*; *C.closterium*-cryopreservation; *N. shiloi*; *N. shiloi*-cryopreservation; *A. capitellata;* *A. capitellata*.

In this study, the specific growth rate of *Amphora cf. capitellata* was determined with the lowest value of 0.1072 ± 0.005 day^{-1} and doubling time of 6.466 day^{-1} whereas the specific growth rate of cryopreserved *A. capitellata* was obtained with the value of 0.2015 ± 0.015 day^{-1}. Dry weights for cryopreserved *Cylindrotheca closterium* and *A. capitellata* were higher than non-cryopreserved cells. Cryopreserved *C. closterium* showed higher lipid productivity than non-cryoreserved *C. closterium* cells. On the other hand, the doubling time and lipid productivity of non-cryopreserved *A. capitella* was two times higher than the cryopreserved *A. capitella* cells. Therefore, cryopreserved *A. capitellata* could not accumulated into fatty acids. In addition, lipid content of *A. capitellata* was obtained at the highest concentration compared to that of the other diatoms. The results were indicated that cryopreserved diatom had higher lipid productivity. The reason is that lipid accumulation has been reported to protect cells from injury during freezing. *Isochrysis galbana* accumulate lipid droplets, which coalesce during cooling and till the cell. During thawing, droplets again become discrete. Hence, the high lipid content of cells could survive under freezing (Grima et al., 1994).

CONCLUSIONS

Cryopreserved diatoms *Amphora cf. capitellata*, *Cylindrotheca closterium*and *Nanofrustulum shiloi* could allow reduction of time consuming and area needed for the maintenance of their live cultures. In the present study, the salt concentration of 1% gave the highest viability for *A. capitellata*, *C. closterium* and *N. shiloi* with 10% Me_2SO for six months in the liquid nitrogen preserved. This could be explained that the low salinity content caused to decrease the penetration of osmotic injury. Therefore, knowledge of the capacity of the cell membrane to lose or gain water osmotically would be useful for more complete understanding of the physical processes affecting cryopreservation of the phytoplanktonic species. Using cryopreservation of marine diatoms to produce fatty acids has several advantages, such as controlled culture conditions, lack of

contamination, and the presence of fatty acids in the polar lipid fraction, and carotenoids as fucoxanthin. Conservation of marine diatoms at sub-zero temperature limiting factors must also be taken into account, such as light, temperature, nutrients, pH, etc. for efficient industrial production of lipids.

ACKNOWLEDGEMENTS. The authors would like to thank the Scientific and Technological Research Council of Turkey (TUBITAK) with the project number of 113Z202 for the financial support.

REFERENCES

Brand, J.J. & Diller, K.R. 2004. Principles and applications of algal cryopreservation. *Nova Hedwigi*a **79**, 175−190.

Bui, T.V.L., Ross, I.L., Jakob, G. & Hankamer, B. 2013. Impact of Procedural Steps and Cryopreservation Agents in the Cryopreservation of Chlorophyte Microalgae. *Plos One* **8**(11) e78668, | www.plosone.org.

Canavate, J.P. & Lubian, L.M. 1994. Tolerance of six marine microalgae to the cryoprotectant dimethyl sulfoxide and Methanol. *Phycol.* **30**(3), 559−565.

Day, J.G. & Brand, J.J. 2005. Cryopreservation Methods for Maintaining Cultures. In: Andersen RA (ed.), *Algal Culturing Techniques*. Academic Press, New York, pp. 165−187.

Day, J.G. & DeVille, M.M. Cryopreservation of algae. *Methods in Molecular Biology* **38**, 81−89.

Day, J.G., Watanabe, M.M., Morris, G.J., Fleck, R.A. & McLellan, M.R. 1997. Long-term viability of preserved eukaryotic algae. *Journal of Applied Phycology* **9**, 121−127.

Day, J.G. & Stacey, G.N. 2007. Cryopreservation and freeze-drying protocols Second edition. *Methods in Molecular Biology* 368, Humana press, Totowa, New Jersey. 141−151.

Demirel, Z., Imamoglu, E. & Conk Dalay, M. 2015. Fatty acid profile and lipid content of *Cylindrotheca closterium* cultivated in air-lift photobioreactor. *J. Chem. Technol. Biotechnol.* **90**(12), 2290−2296.

D'Ippolito, G., Sardo, A., Paris, D., Vella, F.M., Adelfi, M.G., Botte, P., Gallo, C. & Fontana A. 2015. Potential of lipid metabolism in marine diatoms for biofuel production. *Biotechnology for Biofuels*. DOI 10.1186/s13068, 212−224.

Fuller, B.J. 2004. Cryoprotectants: The essential antifreezes to protect life in frozen state. *CryoLetters*. **25**(6), 375−388.

Grima, E.M., Perez, U.J.A., Camacho, F.G., FernBndez, F.G.A., Alonso, D.L. & Segura de Castillo, C.I. 1994. Preservation of the marine microalga, *Isochrysis galbana*: influence on the fatty acid profile. *Aquaculture* **123**, 377−385.

Hedoin, H., Pearson, J., Day, J.G., Philip, D., Young, A.J. & Hall, T.J. 2006. *Porphyridium cruentum* A-408 and *Planktothrix* A-404 retain their capacity to produce biotechnologically exploitable metabolites after cryopreservation. *Journal of Applied Phycology* **18**, 1–7.

Hubalek, Z. 2003. Protectants used in the cryopreservation of microorganisms, Review. *Cryobiology* **46**, 205−229.

Joseph, I., Panigrahi, A. & Chandra, P.K. 2000. Tolerance of three marine microalgae to cryoprotectants dimethyl sulfoxide, methanol and glycerol, *Indian Journal of Marine Sciences* **29**, 243−247.

Levitan, O., Dinamarca, J., Hochman, G. & Falkowski, P.G. 2014. Diatoms: a fossil fuel of the future. *Trends in Biotechnology* **32**(3), 117−124.

Mori, F., Erata, M. & Watanabe, M.M. 2002. Cryopreservation of cyanobacteria and green algae in the NIES-Collection. *Microbiol. Cult. Coll.* **18**(1), 45−55.

Piasecki, B.P., Diller, K.R. & Brand, J.J. 2008. Cryopreservation of *Chlamydomonas reinhardtii*: A cause of low viability at high cell density. *Cryobiology* **58**, 103−109.

Redekar, P.D. & Wagh, A.B. 2000. Cryopreservation studies on the marine diatom *Navicula subinflata* Grun. *Seaweed Res. Utiln.* **22**(1−2), 183−187.

Reed, B.M. 2007. Plant Cryopreservation: A Practical Guide. Spinger, Corvallis, OR, USA, pp. 93−116.
Saks, N.M. 1978. The Preservation of Salt Marsh Algae by Controlled Liquid Nitrogen Freezing. *Cryobiology* **15**, 563−568.
Scholz, B. 2014. Purification and Culture Characteristics of 36 benthic marine diatoms isolated from the Solthörn Tidal Flat (Southern North Sea). *J. Phycol*. **50**, 685–697.
Scholz, B. & Liebezeit, G. 2012. Growth responses of 25 benthic marine Wadden Sea diatoms isolated from the Solthörn tidal flat (southern North Sea) in relation to varying culture conditions. *Diatom Res*. **27**, 65−73.
Tanniou, A., Turpin, V. & Lebeau, T. 2012. Comparison of cryopreservation methods for the long term storage of the marine diatom *Haslea ostrearia* (simonsen). *Cryobiology* 65, 45−50.
Taylor, R. & Fletcher, R.L. 1999. Cryopreservation of eukaryotic algae – a review of methodologies. *Journal of Applied Phycology* **10**, 481−501.
Wood, A.M., Everroad, R.C. & Wingard, L.M. 2005. Measuring growth rates in microalgal cultures. In: Andersen R.A.(ed). *Algalculturing techniques*. USA: Elsevier Academic Press, pp. 269−285.