Indian Journal of Marine Sciences
Vol. 29, September 2000, pp. 243-247

Tolerance of three marine microalgae to cryoprotectants dimethyl sulfoxide, methanol and glycerol

Imelda Joseph*, A. Panigrahi & P. Kishore Chandra
Central Institute of Brackishwater Aquaculture, 101/B, Mahalingapuram, Chennai- 600 034, India

Received 28 January 1999, revised 28 June 2000

Studies on tolerance limit of cryoprotectants, is of much importance in determining the viability of cells after freezing them at below normal temperatures. *Tetraselmis gracilis* showed survival on exposure to 5 to 30% (v/v) DMSO, 5 to 25% methanol and 5 to 40% glycerol for periods of up to 2 h. DMSO and methanol were lethal at higher concentrations (>10%) for *Chlorella marina* and *Chaetoceros calcitrans* even on exposure for 15 min. At 25%, DMSO was so much lethal that the cells of *C. calcitrans* and *C. marina* were dead even after 60 sec. At 20%, all cells were dead within 60 min. Safer concentrations for DMSO were at 5% and lower. Methanol incubations significantly reduced cell viability above 25% (60 sec) for *T. gracilis*, 15% (up to 20 min) for *C. calcitrans* and 20% (up to 60 min) for *C. marina*. All the three algae could tolerate glycerol even up to 30% (60 min). The survival was highest at 10% concentration of glycerol for 120 min. Based on cell recovery rate after 24 h post thaw and growth rate determination, DMSO at 5% or 10% glycerol are the best treatments for cryopreservation. Glycerol was found to be better than DMSO in all the three cases. Post thaw viability in 5% methanol was almost negligent for all the three strains.

Without loss of viability, cryopreserved samples of biological materials have to be stored in liquid nitrogen at –196°C. Much work on cryopreservation in aquatic species is involved in milt samples of various fishes[1]. Cryopreservation of fresh water phytoplankton has also been studied more than that of marine species[2-7]. Since not much information is available on tolerance of cryoprotectants, cryogenic methods used for other species have been tried without detailed studies on their optimization. In the process of cryopreservation, pre-incubation of cells in cryoprotectants is very important in preventing cell damage during freezing. Though, these chemicals are toxic beyond certain levels, their mechanisms are poorly understood[8]. The maximum tolerable limit of cryoprotectant with highest post-thaw viability is used in normal cryopreservation experiments[9].

Cryoprotectants are divided into those that are permeable and non-permeable to cell membrane. The permeable ones reduce the formation of ice and cell volume remains unchanged while freezing as well as thawing. Non permeable cryoprotectants will minimize dehydration of cells during freezing by converting extracellular solution into a gas. Marine micro-algae, living in an environment of high osmonality with high concentrations of non-permeable solutes have a rigid cell wall. This characteristic may result in less biological recovery after shrinking[7]. Therefore, three permeable cryoprotectants, dimethyl sulfoxide (DMSO), methanol and glycerol were selected for the present tolerance of study on three species of marine micro-algae, *Chaetoceros calcitrans, Tetraselmis gracilis* and *Chlorella marina*. The study could help in improving the efficiency of cryopreservation of marine micro-algae and more species could be preserved by this method.

Materials and Methods

Three marine microalgae, *Tetraselmis gracilis* (Prasinophyceae), *Chaetoceros calcitrans* (Bacillariophyceae) and *Chlorella marina* (Chlorophyceae) were selected from the cultures.

Cultures were routinely maintained in F/2 medium[10] in 10 ml test tubes and 100 to 250 ml conical flasks at a temperature of 22 to 25°C and illumination of 800 to 1000 lux on a 12 h: 12 h light and dark cycle. Sub culturing was done in every 10 d. Algae were harvested on 10th day of growth. Harvested algae were centrifuged at 4000 rpm for 5 min. The resulting concentrates were immediately used for the cryoprotectant exposure experiments.

*Present address: NRC of CIBA, Narakkal, Cochin-682 505, Kerala, India.

The salinity of algal concentrates and cryoprotectants were maintained at 30‰. The algae were exposed to 0, 5, 10, 15, 20, 25, 30 and 40% (v/v) concentrations of DMSO, methanol and glycerol. Exposure times were studied by incubating the cultures for 1, 5, 15, 30, 60 and 120 min. All incubations were performed within a temperature range of 22 to 25°C using duplicate test tubes with 1ml of algal suspension and 4ml of cryoprotectant solution. After each incubation time, 0.1 ml of suspension was pipetted out and used to inoculate 10 ml of fresh sterilized F/2 medium in test tubes, achieving 100 times dilution for algae as well as cryoprotectant.

The cells in 10ml test tubes were allowed to grow in the culture conditions described, for 8 to 10 d. The final cell concentration was taken by counting using a hemocytometer and expressed as cells/ml. The samples with different levels of cryoprotectants were observed after 24 h of freezing to record the cell recovery as whole cells remaining after thawing. The viability was calculated from the growth rate achieved in cultures after thawing. For *T. gracilis* and *C. marina,* the determination of cell recovery was difficult, since the non-viable cells did not undergo cell-lysis during freezing. The instantaneous growth rates were calculated as follows:

$$\text{Instantaneous growth rate/d (r)} = \frac{\text{Final count (Ct)} - \text{Initial count (Co)}}{\text{Duration (t)}}$$

For a better and more homogenous graphic comparison of the responses by different algae, the growth rates were expressed as relative growth rates with respect to the unexposed controls.

$$\text{Relative growth rate} = \frac{\text{Final count} - \text{Initial count}}{\text{Initial count}} \times 100$$

The significance of the results obtained in the present study has been validated by two-way ANOVA treatments between concentrations of cryoprotectants and strains.

Results

Algal cultures obtained after exposure to DMSO, methanol and glycerol dilutions showed different responses depending on the species and the concentration of each cryoprotectant (Table 1). The response of *T. gracilis, C. calcitrans* and *C. marina* to different concentrations of glycerol, methanol and DMSO are shown in Fig. 1. Lethal doses of methanol and DMSO took effect mainly between 15 and 30 min. Generally the algae tolerated ascending levels of cryoprotectants up to the level at which viability drastically reduced. Myxotrophic growth was exhibited in some cases where the experimental treatment produced growth responses exceeding the growth of controls (for *C. Calcitrans*, Table 1B), thus showing that some cryoprotectants could enhance growth of algae when present in very little concentrations. *Tetraselmis gracilis* was more tolerant to all the three cryoprotectants at all concentrations (Fig 1). On exposure to DMSO and glycerol, growth was not greater than that for controls even for *T. gracilis*. The highest concentration of DMSO and methanol where cells were viable was 30%, and at 40% it was non-viable. Whereas, *C. calcitrans* at 25% and *C. marina* at 30% cells were non viable in DMSO and methanol. In glycerol, all the three strains were viable up to 30% concentration. While *C. calcitrans* alone was non viable at 40% glycerol. For *C. calcitrans,* rapid mortality was observed from 25 to 40% of methanol and DMSO. The mortality of *T. gracilis* was affected above 10% concentration of methanol. The surviving cells of *T. gracilis* and *C. marina* sedimented at the bottom of the test tube after 24 h incubation (in methanol from 25%). The *C. marina* cells showed a colour change at higher concentrations (20% and above) in DMSO and methanol (bleached). The samples with different levels of cryoprotectants were observed after 24h of freezing. The percentage of cell recovery and growth rate per day after thawing is presented in Table 2. In the present study 5% DMSO and 10% glycerol had enhanced post-thaw viability of cryopreserved micro-algae. On statistical analysis using two way ANOVA, significance in variation was found between three different algal strains over treatments of DMSO, glycerol and methanol at 1% level.

Discussion

The methodology followed in the present study is very much reliable in determining the tolerance of marine micro algae to different cryoprotectants. The methodology followed by Canavate[7] to determine the viability of algal cells after cryopreservation was almost similar to the present one. The cell viability in control after post thaw is less than 0.01% for all the three algal strains. Whereas, the cell recovery and

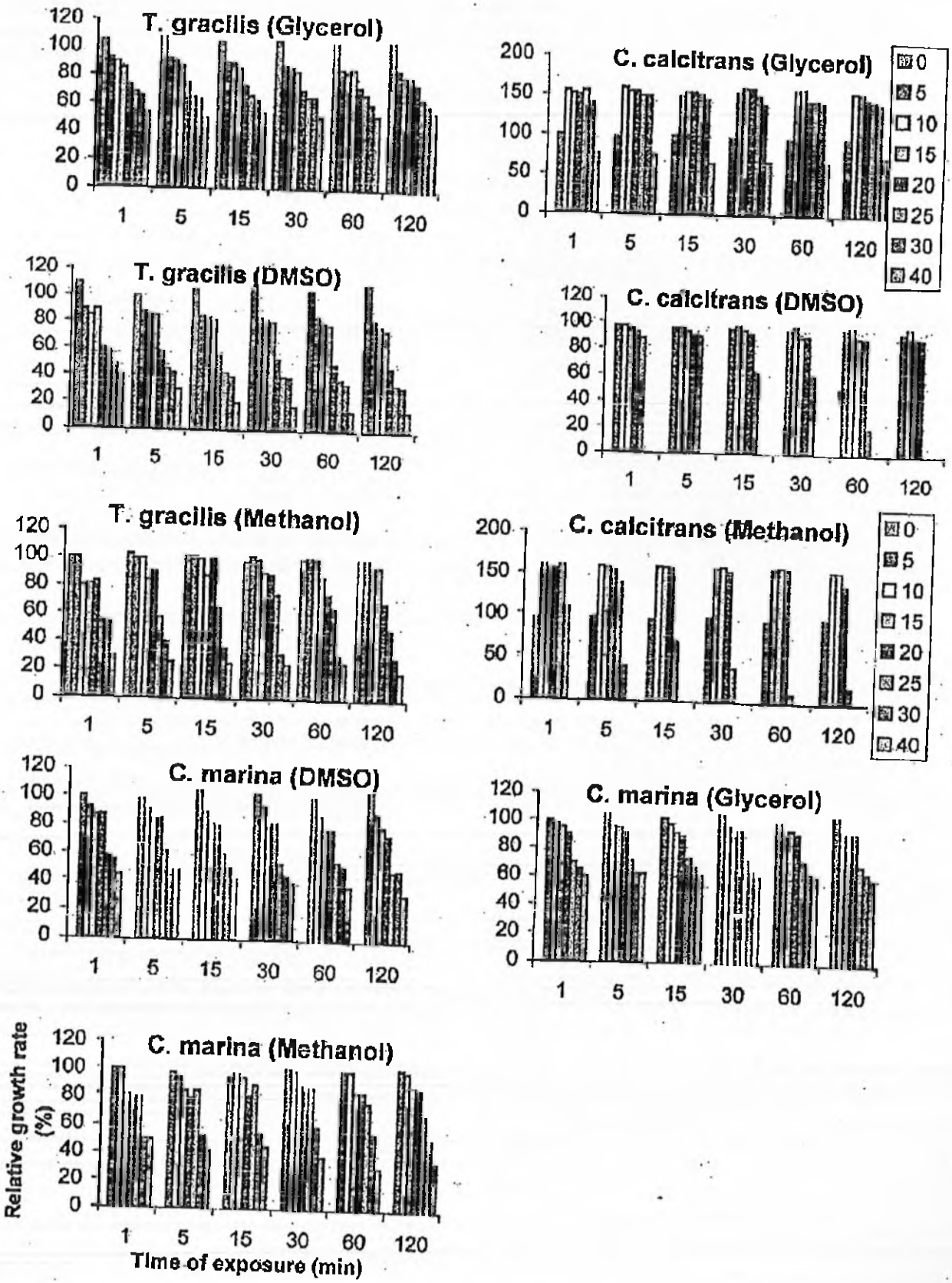

Fig. 1—Relative growth rate of *C. calcitrans*, *T. gracilis* and *C. marina* on using cryoprotectants, DMSO, glycerol and methanol.

Table 1—Final cell density (10^6 cells/ml) and standard deviation (in parentheses) in DMSO, methanol and glycerol concentrations

Algal strain	% Concentrations (v/v) 0	5	10	15	20	25	30	40
				DMSO				
Tetraselmis gracilis	1.0 (0.08)	0.80 (0.03)	0.80 (0.01)	0.81 (0.02)	0.78 (0.05)	0.70 (0.05)	0.30 (0.03)	00
Chaetoceros calcitrans	4.52 (0.28)	4.68 (0.15)	4.30 (0.21)	4.20 (0.12)	1.80 (0.08)	00	00	00
Chlorella marina	8.50 (0.37)	8.30 (0.42)	8.00 (0.28)	7.00 (0.25)	4.0 (0.18)	2.8 (0.08)	00	00
				Methanol				
Tetraselmis gracilis	1.13 (0.16)	0.95 (0.14)	0.82 (0.18)	0.76 (0.20)	0.72 (0.14)	0.56 (0.15)	0.18 (0.18)	00
Chaetoceros calcitrans	4.50 (0.76)	4.90 (0.78)	5.00 (0.65)	4.80 (0.50)	2.50 (0.48)	0.85 (0.43)	00	00
Chlorella marina	9.35 (0.80)	8.00 (0.75)	7.20 (0.70)	6.80 (0.60)	4.50 (0.52)	00	00	00
				Glycerol				
Tetraselmis gracilis	1.20 (0.82)	1.20 (0.58)	1.00 (0.63)	0.98 (0.71)	0.85 (0.80)	0.80 (1.00)	0.40 (0.92)	0.28 (0.11)
Chaetoceros calcitrans	4.50 (0.18)	4.36 (0.16)	4.20 (1.00)	4.00 (0.85)	3.50 (0.90)	2.50 (0.19)	1.00 (0.90)	00
Chlorella marina	9.50 (0.95)	9.40 (1.20)	9.00 (1.13)	8.60 (0.85)	8.00 (0.99)	7.50 (1.5)	5.00 (1.4)	2.00 (1.8)

growth rate for *C. calcitrans* by using 5% DMSO and 10% glycerol was 10.2 to 12.8% and 0.45 to 0.62% respectively (Table 2). No recovery rate was determined for *T. gracilis* and *C. marina*. The growth rate varied from 0.20 to 0.25% in the above treatments of DMSO and glycerol for *T. gracilis*. For *C. marina*, the growth rate ranged from 0.15 to 0.35. Viable cells of *C. calcitrans* could be detected easily after thawing since the damage caused during freezing of cells has reflected in rapid cell lysis, whereas, it was difficult for *T. gracilis* and *C. marina*. For *T. gracilis*, the viable cells were actively moving after 24h of post thaw.

As given in Table 1 (A, B and C), for *C. calcitrans*, *T. gracilis* and *C. marina* 5% DMSO was effective. Glycerol at 5% was good for *C. calcitrans*, *T. gracilis* and *C. marina*, whereas, methanol at 10% was effective only for *C. calcitrans*.

The use of 5% DMSO found to be useful for cryopreservation in the present study as reported by Tsuru[11], whereas, studies by Canavate[7] has shown that algae could grow well after cryopreserving in 15% DMSO. Mc Lellan[4] also had described a 5% (v/v) DMSO concentration as the effective dose for cryopreservation of marine diatoms. Generally DMSO was less effective than glycerol when used as a cryoprotectant at higher concentrations in the present study. Day & Fenwick[5] have also reported a similar observation on cryopreservation of *T. gracilis*.

The loss of cell viability in cells during cryopreservation could be attributed to factors like increase in cryoprotectant level in cells and osmotic changes during ice formations[4,12]. Therefore to assess good cryopreservation of microalgae, its ability to withstand hyper toxicity of cryoprotectants is very important[13,7].

Table 2—Cell recovery and growth rate/d of dilution cultures after thawing from –196°C algal concentrates previously incubated with 5% DMSO, 5% methanol and 10% glycerol and control (without cryoprotectant)

Algal strain	5% DMSO	5% Methanol	10% Glycerol	Control
		T. gracilis		
% Recovery	Nd	Nd	Nd	Nd
Growth rate	0.20	0.00	0.25	0.00
		C. calcitrans		
% Recovery	10.20	0.00	12.80	0.00
Growth rate	0.45	0.00	0.62	0.00
		C. marina		
% Recovery	Nd	0.00	Nd	Nd
Growth rate	0.15	0.00	0.35	0.01

Nd=not determined

The cell structure of *T. gracilis* and *C. marina* remained intact even after 100 fold dilution, whereas, *C. calcitrans* cells were disintegrated on post thaw dilution at lethal levels of cryoprotectants. It was found that methanol is an ineffective cryoprotectant. Mc Lellan[4] has reported a similar observation for diatoms.

An evaluation of algal tolerance to cryoprotectants at controlled room temperatures had not given any indication on loss of viability during the process. The mechanisms of sub-lethal and lethal damage, and the difference in viability among different strains of micro algae has to be investigated further for better understanding of the mechanisms of cryopreservation. As reported in earlier studies, factors such as cooling rate and salinity could also be responsible for the low recovery of *C. calcitrans* compared to *T. gracilis* and *C. marina*[5,14].

Acknowledgement

We are thankful to Dr G R M Rao, Director, Central Institute of Brackishwater Aquaculture (CIBA), Chennai and Dr L. H. Rao, Head of Division, Crustacean Culture Division, for the constant encouragement during the present study.

References

1 Leung L K P & Jamieson B G M, Live preservation of fish gamates. In: *Fish evolution and systematics: Evidence for spermatozoa*, edited by B.G.M. Jamieson (Cambridge University Press, Cambridge) 1991, pp. 245-265.

2 Beaty M. H. & Parker B C, Investigations of cryopreservation and storage of eukaryotic algae and protozoa, *J Phycol*, 26 (Suppl) (1990) 5.

3 Morris G M, Cryopreservation of 250 strains of *Chlorococcales* by the method of two-step cooling, *Brit Phycol J*, 13 (1978) 15-24.

4 Mc Lellan M R, Cryopreservation of diatoms, *Diatom Res*, 4 (1989) 301-318.

5 Day J G & Fenwick C, Cryopreservation of members of the genus *Tetraselmis* used in aquaculture, *Aquaculture*, 118 (1993) 151-160.

6 Grima E M, Sanchez Perez J A, Camacho F G, Acien Fernandez F G, Lopez Alonso D & Segura del Caslillo C I, Preservation of the marine alga, *Isochrysis galbana*: Influence on the fatty acid profile, *Aquaculture*, 123 (1994) 377-385.

7 Canavate J P and Lubian L M, Tolerance of six marine micro algae to the cryoprotectants dimethyl sulfoxide and methanol, *J Phycol*, 30(1994) 559-565.

8 Fahy G M, Lilley T H Lindsell H, Douglas M J & Meryman H T, Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms, *Cryobiology*, 27 (1990) 247-268.

9 Morris G J & Farrant T, Interactions of cooling rate and protective additive on the survival of washed human erythrocytes frozen to –196°C, *Cryobiology*, 9 (1972) 173-181.

10 Guillard R R L & Ryther J H, Studies on marine planktonic diatoms:I. *Cyclotella nana* Hustedt and *Detinula conferacea* (Cleve) Gran, *Canadian J Microbiol*, 8 (1962) 229-239.

11 Tsuru S, Preservation of marine and freshwater algae by means of freezing and freeze-drying, *Cryobiology*, 10 (1973) 445-452.

12 Morris G M, The cryopreservation of *Chlorella*. I. Interactions of rate of cooling, protective additive and warming rate, *Arch Microbiol*, 107 (1976) 57-62.

13 Morris G M, in: *Cryopreservation* (Institute of Terrestrial Ecology, Cambridge) 1981, pp 1-27.

14 Montaini E, Zittelli G C, Tredici M R, Grima E M, Fernandez Sevilla J M & Sanchez Perez J A, Long term preservation of *Tetraselmis suecica*: Influence of storage on viability and fatty acid profile, *Aquaculture*, 134 (1995) 81-90.