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Research Article

Cryopreservation of the microalgae *Chaetoceros calcitrans* (Paulsen): analysis of the effect of DMSO temperature and light regime during different equilibrium periods

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ABSTRACT. We evaluated the effect of three variables (cryoprotectant temperature, light regime, and time of exposure to the cryoprotectant) throughout the equilibrium period during cryopreservation on the viability of the microalga *Chaetoceros calcitrans* (Paulsen). For this, the cryoprotectant dimethyl sulfoxide (DMSO) at 5% (v/v) was added at three different temperatures (4, 10, and 25ºC) before placing the microalgae in cryobiological straws for freezing. Once inside the cryobiological straws, the microalgal-cryoprotectant suspensions were subjected to the following light regimes for 15 or 45 min: complete light, complete darkness, light/darkness, and darkness/light. Suspensions were then frozen under controlled conditions and stored in liquid nitrogen. The viability index proposed by Cañavate & Lubian (1995b) was used to measure microalgal viability after cryopreservation. Results indicated that it is necessary to use a cryoprotectant to ensure the viable cryopreservation of *C. calcitrans*. Statistical analyses showed that the temperature of the DMSO influenced the viability of cryopreserved microalgae and that there was no synergistic effect between the other variables studied. The viabilities obtained with DMSO at 25ºC, 10ºC, and 4ºC were 34.9%, 27.8%, and 20.6%, respectively.

Keywords: cryopreservation, diatoms, DMSO, temperature, light, equilibrium period.

Criopreservación de las microalgas *Chaetoceros calcitrans* (Paulsen): análisis del efecto de la temperatura de DMSO y régimen de luz durante diferentes períodos de equilibrio

RESUMEN. Se evaluó el efecto de tres variables – la temperatura del crioprotector, el régimen de luz y el tiempo de exposición al crioprotector durante el período de equilibrio en el proceso de criopreservación – sobre la viabilidad de la microalga *Chaetoceros calcitrans* (Paulsen). Para ello se utilizó, el crioprotector dimetilsulfóxido (DMSO) al 5% (v/v), el cual fue adicionado a tres temperaturas diferentes (4, 10 y 25ºC) antes de que las microalgas fueran introducidas dentro de pajuelas criobiológicas para su congelación. Una vez dentro de las pajuelas, las suspensiones de microalgas con crioprotector se sometieron durante 15 o 45 min a luz completa, oscuridad completa, luz/oscuridad y oscuridad/luz. Luego se congelaron de manera controlada y se almacenaron en nitrógeno líquido. La viabilidad de las microalgas posterior a la criopreservación fue medida a través del índice de viabilidad propuesto por Cañavate & Lubian (1995b). Los resultados indicaron que es necesario el uso de criprotector para la criopreservación viable de *C. calcitrans*. El análisis estadístico demostró que la temperatura del DMSO influye sobre la viabilidad de la microalga criopreservada y que no existe efecto sinérgico entre las demás variables de estudio. Las viabilidades alcanzadas usando DMSO a 25ºC, 10ºC y 4ºC fueron de 34,9%, 27,8% y 20,6% respectivamente.

Palabras clave: criopreservación, diatomea, DMSO, temperatura, luz, periodo de equilibrio.

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INTRODUCTION

Microalgae used in aquaculture offer main nutritional requirements to vertebrate and invertebrate aqua-cultures. For example, species such as *Chaetoceros calcitrans* offers high content of fat acids (EPA), antioxidants e immune system stimulants substances (vitamin C and B2), and its small size (2.5 to 6 µm of

diameter) makes it strongly appropriated to feed mollusks, echinoderms, crustaceans and some zooplankton like *Artemia* (Brown *et al*., 1997, 1999; Becker, 2004; Krichnavarruk *et al*., 2005; Khoi *et al*., 2006).

The intensive production of microalgae for feeding purposes, often face problems caused by loss of stock cultures or nutritive quality of these microorganisms (Cañavate & Lubian, 1995a; Day & Brand, 2005; Mitbavkar & Chandrashekar, 2006; Gwo *et al*., 2005).

For that reason, aquaculture facilities always look for strains of microalgae − from subcultures or cryopreserved collections − with biochemical profiles suitable for feeding commercial species (Cañavate & Lubian, 1995b; Cordero & Voltolina, 1997; Day & Harding, 2007).

Cryopreservation allows the viable storage of cells or tissues over long periods of time at extremely low temperatures (-196ºC), keeping them in a state of "suspended animation" in which biological activity practically stops (Saks, 1978). The main advantages of microalgal cryopreservation are the immediate availability of strains for culture, low costs of maintenance of strains, optimal use of space and materials, and avoidance of genetic drift that could occur through serial transfer when cultures are maintained long-term (Kuwano *et al*., 2004). Despite the importance of cryopreservation, its application to microalgae should be studied to better understand the degrees of susceptibility and sensitivity of these organisms to cryopreservation, such as: type of strain and the effect of cell size, shape, growth and rate phase, freezing and thawing rate, storage temperature and duration, among others, that affect post-freezing viability (Mortain-Bertrand *et al*., 1996; Taylor & Fletcher, 1999; Rhodes *et al*., 2006; Day & Harding, 2007).

Cryopreservation involves three stages: equilibrium, freezing and thawing. To protect cells throughout the process, cryoprotectant agents (CPAs) are added before freezing (Fuller, 2004; Dumont *et al*., 2006). During the equilibrium period, the microalgae-cryoprotectant suspension is maintained for a predefined time to allow exchange between intracellular solutes and the cryoprotectant solution. Three important variables are related to the use of CPAs: concentration, temperature and time of exposure, and there are different methods of handling them reported in literature. For example, tests with DMSO concentrations ranging from 1 to 32%, equilibrium periods lasting from 10 to 240 min and temperatures ranging between 4º and 23ºC (Panis *et al*., 1990), have indicated that low CPA concentrations (< 10%) and short equilibrium periods (< 30 min) at low temperatures (0-10ºC) increase post-thawing viability (Tzovenis *et al*., 2004; Day, 2007). Other studies suggest that high viability can be obtained with concentrations of approximately 20% CPA, equilibrium periods between 30-45 min (or longer) and temperatures of approximately 20ºC (Cañavate & Lubian, 1994, 1995b, 1997a, 1997b; Poncet & Verón, 2003; Mitbavkar & Chandrashekar, 2006; Gwo *et al*., 2005). These contrasting results have not only been reported in microalgae, but also in other microorganisms (Hubálek, 2003; Wu *et al*., 2008). Another important aspect during the equilibrium period is the chemical and osmotic stress induced by the addition of CPAs. Under stressed conditions, the light irradiation of cells can damage the photosynthetic systems (photosystem II). This damage can be lethal, depending on the amount of time cells are exposed to stress, even if it is to low-light irradiation (Yordanov & Velikova, 2000; Murata *et al*., 2007).

Several microalgae species have been cryopreserved with different methodologies obtaining viabilities up to 100% (Cañavate & Lubian, 1994, 1995a , 1995b). In the case of species of the genera *Chaetoceros*, *Chaetoceros gracilis* (Cañavate & Lubian, 1995b, 1997a) and *Chaetoceros* sp. (Cordero & Voltolina, 1997) have shown viabilities after cryopreservation lower than 40%. For the species *Chaetoceros muelleri*, cryopreservation has been successfully reported using DMSO 10% and 15%, however, there are not quantitative reports of viability (Rhodes *et al*., 2006).

On the basis of the information mentioned above, this study aims to analyze the effect of the DMSO temperature, time of exposition to this cryoprotectant and light regimen during the equilibrium period on the post-cryopreservation viability of *C. calcitrans*.

MATERIALS AND METHODS

Culture of Chaetoceros calcitrans

A strain of *C. calcitrans* was obtained from the laboratory of Universidad Católica del Norte at Coquimbo, Chile. Two flasks of 250 mL were used to establish two 120 mL cultures using Guillard & Ryther (1962) medium enriched with silicate (30‰ $Na_2SiO_3.9H_2O$). Cultures non axenic but free of protozoan and bacteria (genera *Vibrio*) were maintained at 20ºC, under continuous irradiation at 65 µmol photons m^{-2} s^{-1} provided with cold light lamps (Philips TL-TRS 40W/54). One culture was labeled as *t* and the other, as *d*. Both *t* and *d* cultures were

harvested on the 5th day. The *t* culture was carried to intermediate culture with a volume of 1400 mL under the conditions of culture media, temperature, and irradiation (same conditions above mentioned). The *d* culture, was used to estimate a growth curve using different initial densities. Seven cultures of 120 mL were inoculated with approximately 95000 (*d1*), 360000 (*d2*), 865000 (*d3*), 970000 (*d4*), 1310000 (*d5*), 1585000 (*d6*) and 2565000 (*d7*) cells per mL^{-1}. Growth according to initial and final densities was analyzed based on a function of potential correlation with the equation $Y = aX^b$ (Cañavate & Lubian, 1995b).

The *t* culture was used to perform cryopreservation tests. To begin treatments, the intermediate *t* culture was harvested on the 6th day and placed in 50 mL tubes, which were then centrifuged at 2500 rpm for 10 min. The microalgal pellet obtained was resuspended and distributed into three 50 mL tubes to carry out all treatments (each tube was considered a replicate of the entire experiment and also was used to make its own control).

Cryopreservation procedures

To apply treatments, 0.30 mL microalgal concentrate from intermediate *t* culture were placed in 1.5 mL Eppendorf tubes. The stock solution of the cryoprotectant dimethyl sulfoxide (DMSO) was prepared at 10% (v/v) with autoclaved and micro filtered seawater and it was added at a ratio of 1:1 (microalgae:cryoprotectant) to each sample at three temperatures (4, 10, and 25ºC). Final cryoprotectant concentration was 5% (v/v). At each temperature, the microalgae-cryoprotectant suspensions were introduced into 0.5 mL straws (IMV France) and sealed with polyvinyl alcohol (Sigma). The equilibrium period lasted 15 or 45 min while the straws were exposed to four light regimes: complete light, complete darkness, light/darkness and darkness/light. Light and darkness in the latter two regimes were distributed equally as follows: for the 15 min period, straws with microalgae were exposed to 7.5 min light and 7.5 min darkness and vice versa; for the 45 min period, straws were exposed to 22.5 min light and 22.5 min darkness and viceversa (Table 1). Light irradiation in each treatment was the same mentioned in culture conditions.

To compare the effect of cryopreservation with and without DMSO, additional tests were carried out at temperatures of 4, 10, and 25°C and with 15 min of equilibrium period under complete light. All straws were filled as described before but sterilized microfiltered seawater was added instead of the cryoprotectant.

All tests were carried out using a CryoLogic CL-3300 freezing system and the CryoGenesis™ V5 software. The temperature of the straws was diminished starting at treatment temperatures (4, 10, and 25ºC) down to -4ºC, using a cooling rate of 3ºC min^{-1}. This temperature was held for 1 min and continuing with the freezing at the same cooling rate down to -60ºC (held for 10 min). Finally, the straws were placed in liquid nitrogen (-196ºC) and stored until evaluation.

Samples were thawed using a water bath at 25ºC for 2 min and the content of the straws was distributed in 1.5 mL Eppendorf tubes. Culture medium was added step-by-step to a 0.1 mL aliquot of microalgae-cryoprotectant solution of each treatment in tubes. Each tube was used to carry out a 10 mL culture under the same growth conditions described previously. Cell density was measured every day from the inoculation day until day 5 using a Neubauer counting chamber under a phase-contrast photon microscope (Nikon Optiphot).

Evaluation of growth and viabilities

Culture growth curves were estimated using exponential correlation. Specific growth (μ) of *C. calcitrans* was calculated using the following equation:

$$\mu\ (day^{-1}) = [\mathrm{Ln}(C_f/C_i)]/(t_f - t_i) \quad (1)$$

where C_i is the initial cell concentration; C_f, the final cell concentration; and t, the time of culture in days. Specific growth (μ) was calculated after the adaptation phase. Growth curves were illustrated for each treatment according to Affan *et al.* (2007).

The viability index (V) proposed by Cañavate & Lubian (1995b) was used to estimate post-cryopreservation viability:

$$V = [(C_0/C_i)\mathrm{x}(C_5/(\mathrm{a}C_0{}^{\mathrm{b}})] \text{ x } 100 \quad (2)$$

where C_0 is the inoculation density (day 0); C_5, final density for day 5 of culture; C_i, maximum initial density (dilution 1:100 prior to cryopreservation); *a* and *b*, are regression coefficients obtained for 5 days in relation to C_0 in the *d* cultures.

The term $aC_0{}^b$ is equivalent to the expression aX^b in a potential equation.

The experiment corresponded to a completely randomized factorial design that generated 24 treatments (Table 1), each with three replicates. Three non-cryopreserved controls and *d* cultures were available throughout the process. Viability indexes (V) were calculated for each replicate and were compared using a factorial ANOVA, followed by Duncan's

Table 1. Light regime, temperature, and time of exposure of microalgae to DMSO during the equilibrium period for the different treatments (T).

Tabla 1. Régimen de luz, temperatura y tiempo de exposición de las microalgas durante el tiempo de equilibrio para los diferentes tratamientos (T).

Light regime	Complete light			Complete darkness			Light/darkness			Darkness/light		
Time of exposure (min)	DMSO temperature (°C)											
	4	10	25	4	10	25	4	10	25	4	10	25
15	T1	T9	T17	T2	T10	T18	T3	T11	T19	T4	T12	T20
45	T5	T13	T21	T6	T14	T22	T7	T15	T23	T8	T16	T24

multiple range tests. All statistical analyses were performed with a 95% confidence level (α = 0.05) using Statsoft statistics software version 7 (Statsoft, UK).

RESULTS

Non-cryopreserved cultures (controls)

Cell growth was similar in controls of all replicates and there were no significant differences among them (P > 0.05). Growth curves showed correlation coefficients ranging between 0.86 and 0.89. Average initial density and on the fifth day were 2.0 x 10^6 cells mL^{-1} and 3.5 x 10^6 cells mL^{-1}respectively. Controls and d cultures showed similar growth tendencies ($Y = ae^{bx}$). The specific growth rate was faster in cultures obtained from inoculations with low cell density mL^{-1} (Table 2). The correlation aX^b for *d* cultures indicated an inverse relationship between initial and final inoculation densities ($Y = 1 \times 10^6 X^{-0.072}$; $R^2 = 0.8325$).

Cryopreservation of *Chaetoceros calcitrans*

Post-cryopreservation cultures

All cryopreservation tests without DMSO at 4, 10 and 25ºC, resulted in complete cell death on day 2 of culture post-cryopreservation. In those treatments using DMSO, different percentages of viability were observed after thawing (day 5).

Initial culture densities of post-cryopreservation treatments at time of inoculation (C_0) ranged between 3.1 x 10^5-7.7 x 10^5 cells mL^{-1}, and densities on day 5 of culture (C_5) varied between approximately 2.5 x 10^6-3.7 x 10^6 cells mL^{-1}. Growth curves of the 24 treatments exposed to cryopreservation exhibited an adaptation phase within the first two days of culture. High cell death and low densities were observed on day 2 in all cultures. From day 3 on, evidence of an exponential growth phase was determined from a high cell growth rate (Fig.1).

Specific growth (μ day^{-1}) was higher in cultures of cryopreserved cells than in non-cryopreserved cells (controls). Growth curves of treatments, as well as, specific growth rates were similar to those reported for *d* cultures. The highest specific growth rates in cryopreserved treatments were reached with the treatment with the lowest cell densities (treatments 17, 15, and 13) and conversely, low specific growth rates were reached with treatments with higher cell densities (treatments 22, 23, and 18) (Table 2). Of the treatments with DMSO at 4 and 10ºC, 93.75% presented average growth rates above 1 (μ day^{-1}), whereas only 50% of the treatments with DMSO at 25ºC exceeded that same growth rate. Controls presented a growth rate of 0.11 μ day^{-1}.

Estimation and comparison of viability

The average initial concentration (C_i) before cryopreservation was of 2.0 x 10^6 cells mL^{-1}. The viability of *C. calcitrans* in all cases was higher than 12.2% (treatment 15) and did not exceed 34.9% (treatment 22) (Table 2, Fig. 2). A factorial ANOVA was performed to identify those variables that most influence the viability of cryopreserved *C. calcitrans*. Data (proportion of viability) presented a normal frequency distribution according to the Kolmogorov-Smirnov test (P > 0.05), and variance homogeneity was verified according to Levene's test (P > 0.05). Hypotheses of the statistical model and their interactions were compared by parametric statistical analysis, which indicated that only the temperature of DMSO had a significant effect (P < 0.05) on the viability of cryopreserved *C. calcitrans* (Table 3). Duncan multiple range analysis, performed after the factorial ANOVA, revealed significant differences between the temperatures of 4 and 25ºC and between 10 and 25°C (P < 0.05), whereas there were no significant differences between the temperatures of 4 and 10°C. Although analyses indicated that the light regimen had no statistically significant effect, average

Table 2. Growth rates (μ day^{-1}) and viabilities indexes of *Chaetoceros calcitrans.* Controls, *d* cultures and treatments.

Tabla 2. Tasas de crecimiento (μ dia^{-1}) e índices de viabilidad para *Chaetoceros calcitrans.* Controles, cultivos *d* y tratamientos.

Treatment	μ day^{-1}	Viability index (%)	Treatment	μ day^{-1}	Viability index (%)
Control	0.11 ± 0.04	–	9	1.16 ± 0.22	21.5 ± 2.72
d1	1.28*	–	10	1.25 ± 0.49	21.1 ± 12.78
d2	0.83*	–	11	1.05 ±0.32	22.9 ± 3.53
d3	0.51*	–	12	1.30 ± 018	21.5 ± 11.10
d4	0.47*	–	13	1.41 ± 0.19	14.1 ± 0.89
d5	0.35*	–	14	1.01 ± 0.35	27.8 ± 17.55
d6	0.28*	–	15	1.58 ± 0.04	12.2 ± 3.08
d7	0.10*	–	16	1.10 ± 0.45	19.5 ± 12.54
1	1.10 ± 0.13	19.1± 6.90	17	1.67 ± 0.30	16.7 ± 11.57
2	1.28 ± 0.41	15.5 ± 7.64	18	**0.75 ± 0.23**	**31.7 ± 7.71**
3	1.20 ± 0.25	18.5 ± 9.89	19	1.03 ± 0.19	29.0 ± 10.60
4	1.15 ± 0.38	20.6 ± 1.94	20	0.88 ± 0.06	23.5 ± 4.73
5	0.88 ± 0.15	19.3 ± 13.75	21	1.09 ± 0.17	14.8 ± 6.53
6	1.16 ± 0.23	15.6 ± 8.44	22	**0.70 ± 0.03**	**34.9 ± 7.82**
7	1.03 ± 0.18	16.6 ± 9.25	23	**0.77 ± 0.08**	**32.7 ± 7.84**
8	1.09 ± 0.28	16.0 ± 4.96	24	1.04 ± 0.38	21.4 ± 14.43

*single measurements

Data highlighted in bold indicates the highest viabilities.

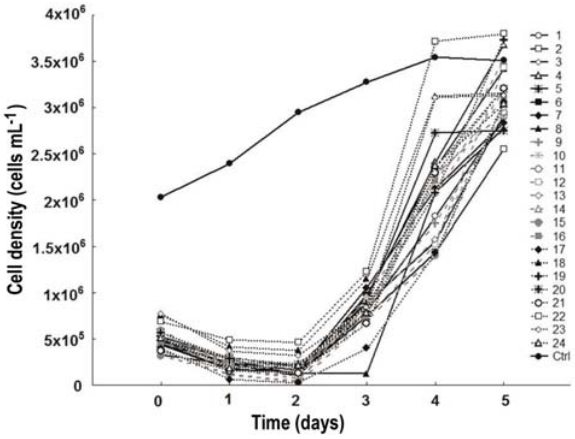

Figure 1. Growth curves of all treatments exposed to cryopreservation (numbers to the right indicate treatments; Ctrl: control).

Figura 1. Curvas de crecimiento de todos los tratamientos sometidos a criopreservación, (Números a la derecha: indica el tratamiento y Ctrl: control).

viability tended to increase with complete darkness and a 15 min of time of exposure.

DISCUSSION

Growth and cryopreservation

Cell growth of *Chaetoceros calcitrans* was similar in both *d* and *t* cultures (prior to cryopreservation). No adaptation phase was detected in either culture. If it did occur, it was very mild, similar to that reported by Phatarpekar *et al*. (2000) for the same species; this could be attributed to faster cellular reproduction than in other species. For example, adaptation phases longer than 1 day have been reported for *Chaetoceros muelleri* and *C. gracilis, Tetraselmis tetrathele* and *T. gracilis, Dunaliella salina* and *D. tertiolecta,* and *Navicula incerta,* among others (Calderón & Serpa, 2003; Leal *et al*., 2003; Affan *et al.*, 2007; Moura-Junior *et al*., 2007). The specific growth rates of *d* cultures were higher with low densities of inoculated cells as it was expected; since they are conditioned by factors such as nutrient depletion, pH alteration, CO_2 deficiency, and reduced light penetration, among others (Fogg & Than-Tun, 1960).

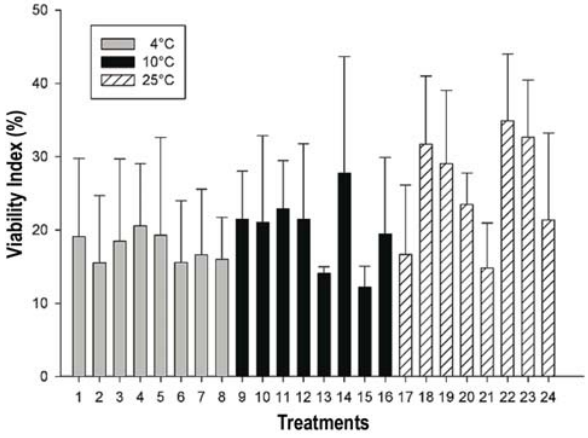

Figure 2. Effect of temperature of DMSO (5%) during the equilibrium period on viability of *Chaetoceros calcitrans*.

Figura 2. Efecto de la temperatura del DMSO (5%) durante el período de equilibrio sobre la viabilidad de *Chaetoceros calcitrans*.

It was only possible to generate viable cell cultures of *C. calcitrans* when DMSO was added. Low or no viability has been reported for the species *C. gracilis* (Cañavate & Lubian, 1995b), *Chaetoceros* sp. (Cordero & Voltolina, 1997), and *Nannochloropsis oculata* (Gwo *et al*., 2005) in the absence of CPA. Although viabilities up to 69% have been obtained without using CPA for *Tetraselmis chuii*, *Nannochloropsis gaditana* and *Nannochloris atomus* (Cañavate & Lubian, 1995b) and, certain Chlorococcaceae and Cyanobacteria (Day *et al*., 2000). Such differential responses to cryopreservation (with/without cryoprotectant) depend directly on each species physiological mechanisms to tolerate osmotic stress induced by below-zero temperatures. In the case of *C. calcitrans*, its reaction to cryopreservation without DMSO could be explained by the excessive dehydration at the intracellular level caused by the formation of extracellular ice crystals that promote cell death (Tanaka *et al*., 2001; Day & Harding, 2007).

Table 3. Factorial variance analysis (ANOVA) of viability post-cryopreservation of *C. calcitrans*. SS: sum of squares. DF: degrees of freedom. SM: square of the mean, and F values and *P* value.

Tabla 3. Análisis de Varianza Factorial (ANOVA) de la viabilidad posterior a la criopreservación de *C. calcitrans*. SS: Suma de cuadrados. DF: grados de libertad. SM: Cuadrado de la media, F y *P* (estadísticos).

Variable	SS	DF	SM	F value	P value
Temperature (DMSO)	0.079	2	0.039	4.638	0.014
Light regime	0.044	3	0.014	1.735	0.172
Time of exposure	0.003	1	0.003	0.408	0.525
Temperature x light regime	0.094	6	0.015	1.841	0.110
Temperature x time of exposure	0.004	2	0.002	0.290	0.749
Light regime x time of exposure	0.013	3	0.004	0.521	0.669
Temperature x light regime x time of exposure	0.019	6	0.003	0.375	0.891
Error	0.411	48	0.008		

Mechanical damage, cannot be discarded because several organelles could have been destroyed. The responses to cryopreservation obtained in this study, as well as the results of Cordero and Voltolina (1997) in *Chaetoceros* sp., suggest that the presence of CPAs is necessary for post-freezing cell recovery in *Chaetoceros* microalgae as in other microorganisms. DMSO has shown high levels of toxicity in several species (Cañavate & Lubian, 1994; Fuller, 2004). Although in the case of *C. calcitras* was indispensable to obtain viable cells after criopereservation, which indicates a natural tolerance to this kind of substances in its cytoplasm. Natural tolerance for freezing and hiperosmotic substances in other diatoms of this genera, such as *Chaetoceros castracanei* and species from Artic and Antartic, has been attributed to the accumulation of free amino acids (mainly proline) and DMSP (a DMSO precursor) (Ferrario *et al*., 2004; Mock & Thomas, 2005; Houdan *et al*., 2005; Ralph *et al*., 2005).

Effectiveness of cryopreservation

The maximum mean viability reached in this study was 34.9%. In studies conducted by other authors (Cañavate & Lubian, 1995b, 1997a, 1997b), the viabilities of different non-diatom microalgae treated with CPA reached 100%, whereas viabilities for diatoms such as *C. gracilis* were below 40%, this suggests that, within the physiological mechanisms of diatoms to respond to osmotic stress, the presence of a rigid silicon wall seems to play an important role in cryopreservation because it could confer fragility to

the cryopreserved cell (rupture during freezing or thawing). Mortality caused by that fragility could be minimized, in the case of *C. calcitrans*, by the high surface/volume ratio of this species, which, would help reduce the formation of intracellular ice (Mortain-Bertrand *et al*., 1996). However, this should not be considered a rule for small-sized diatoms because other factors, such as rate of freezing and thawing, also affect microalgae viability, regardless of the inherent conditions of the cells and growth conditions (Ben-Amotz & Gilboa, 1980a, 1980b; Cañavate & Lubian, 1997a, 1997b; Day *et al*., 1998).

The analysis of the variation of V indicated that the temperature of DMSO during the equilibrium phase is the factor that most influences post-freezing viability of *C. calcitrans*. The Duncan multiple range test indicated that viability at 4 and 10ºC is lower than at 25ºC. Based on these results, the optimal temperature of DMSO during the equilibrium phase of cryopreservation of *C. calcitrans* is 25°C. This temperature is close to the range mentioned by Taylor & Fletcher (1999), who recommended using equilibrium temperatures between 18 and 23°C, as well as to that proposed by other authors (Cañavate & Lubian, 1995b,1997a, 1997b), who obtained high viabilities (> 90%) with different microalgae species. Studies conducted by Tanaka *et al*. (2001) also indicated that temperatures close to 23ºC facilitate the osmotic exchange of DMSO solution and the cell during the equilibrium period, because increasing temperatures increase molecular movement and kinetic energy of the H_2O-DMSO solution; this causes DMSO diffusion to proceed faster at 25ºC with the appropriate solute concentration than at 4 and 10ºC. In this study, when DMSO was added at 4 and 10°C, average V were lower than 20%, contrary to what was found by other authors (Panis *et al*., 1990; Mortain-Bertrand *et al*., 1996; Hubálek, 2003) who suggested that temperatures between 0 and 10°C during the equilibrium period are appropriate for cryopreservation because they protect the cells from CPA toxicity since at physiological temperatures, the cryoprotectant is highly toxic. We suggest that it is more important to ensure the entry of DMSO into the cell than to try to minimize its toxicity because the freezing/thawing process apparently has a more traumatic effect than that induced by CPA toxicity.

In addition to the temperature and concentration of DMSO, the length of the equilibrium period is also an important aspect to be considered. In this study, statistical analyses indicated that the length of two equilibrium periods (15 and 45 min) with DMSO does not have a significant effect on the viability of *C. calcitrans*. This can be attributed to DMSO is a fast-penetrating cryoprotectant and does not require a prolonged equilibrium period to yield good results, as compared with slow-penetrating CPAs, such as glycerol, that need more than 60 min (Karlsson & Toner, 1996; Day *et al*., 1998; Hubálek, 2003; Fuller, 2004). The DMSO rate of penetration during the equilibrium period depends on cell size and type, differential permeability of cell membranes, and membrane lipid content. Therefore the lengths of effective equilibrium periods vary from species to species (Salinas-Flores *et al*., 2008). Norton & Joiner (1968) reported DMSO 7.5% and equilibrium periods up to 149 min to cruopreserve sporocites of *Eimeria tenella* due its low permeable membrane.

There are no reports on the effect of using light regimens during the equilibrium period similar those used in this study. The light regime results did not lead to the detection of significant differences in viability; however, it is interesting to note a pattern of low viabilities seen in those treatments that included "complete light". This result could be attributed to alteration or damage in the electron transport system. This has been described for the green algae *Chlamydomonas reinhardtii* where severe damage was observed in the electron transport chain when the algae was exposed to methanol in the presence of light during the equilibrium period. Piasecki *et al*. (2009) recommend that these microorganisms be cultured under very low light or in complete darkness during this phase.

Finally, post-cryopreservation cell survival or mortality can be mainly attributed to the existence of several levels of genetic variability (Medlini *et al*., 2000), which would be responsible for the differential response of *C. calcitrans* cells to osmotic stress and post-freezing survival lower than 100% (Fahy, 1986).

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