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Note

A Modified Cryopreservation Method of Psychrophilic Chlorophyta *Pyramimonas* sp. from Antarctica

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Abstract : Polar psychrophiles which thrive under extreme conditions such as cold temperature, high salinity, and high dose ultraviolet light, emerge as novel targets for biotechnology. To prevent genetic drift and the possibility of contamination by subculturing, cryopreservation was employed for two psychrophilic microalgae, *Porosira* sp. (KOPRI AnM0008) and *Pyramimonas* sp. (KOPRI AnM0046), which have antifreeze activities. Five cryoprotectants (dimethyl sulphoxide, ethylene glycol, glycerol, methanol and propylene glycol) showed toxicity at 20-30% (v/v). The optimal cryoprotectant concentration and equilibration time were less than 20% and 10 min, respectively. Cryopreservation was carried out in the presence of cryoprotectants either by direct freezing in liquid nitrogen (LN_2) or controlled freezing using a controlled rate freezer followed by storage in the LN_2 tank. As a result, *Pyramimonas* sp. (KOPRI AnM0046), a psychrophilic chlorophyta was revived. Cryopreserved *Porosira* sp. was not revived from either freezing protocols probably due to the silicic cell wall and its relatively large cell size. In the case of *Pyramimonas* sp. (KOPRI AnM0046), the controlled freezing method showed higher revival yield than the direct freezing method.

Key words : cryopreservation, psychrophile, microalgae, Pyramimonas, antifreeze activity

1. Introduction

A psychrophile is an organism reproducing and growing optimally at low temperatures, specifically, ranging from −10 to 20°C. They inhabit in perennially cold environments, such as deep sea, permafrost, polar and alpine regions, sea ice and so on (Margesin and Feller 2010). Like thermophiles, psychrophiles are also emerging as novel targets for biotechnology. Adaptation mechanisms of psychrophiles to cope with low temperatures, high salinity, and high dose ultraviolet light have led to features including cold active enzymes, antifreeze proteins, modulation in membrane fluidity, expolysaccharides etc. (Margesin and Feller 2010).

Among them, antifreeze proteins (AFPs) have drawn interests from various fields such as food technology, preservation of cell lines, organs, cryosurgery, and cold resistant transgenic plants and animals, because it may reduce the freeze injuries during freezing (Venketesh and Dayananda 2008). AFPs are a group of proteins that bind to ice and inhibit the growth of ice crystals upon cooling below the bulk melting point. They were first found in Antarctic fish and were known to lower the freezing point of blood serum of polar fish, thus allowing them to survive in subzero environments (DeVries et al. 1970). They have also been identified in insects, plants bacteria, fungi, yeast, and algae (See references in Venketesh and Dayananda 2008). Recently we have found that *Pyramimona*s sp. (KOPRI AnM0046), a chlorophyta and

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Porosira sp. (KOPRI AnM0008), a diatom from our Korea Culture Collection of Polar Microorganisms (KCCPM) displayed antifreeze activities, meaning that they produce antifreeze proteins. These psychrophilic microalgae have great biotechnological potential and we, therefore, need to adequately preserve them for a long term period without losing their unique traits.

Presently, KCCPM microalgae populations have been maintained by serial cultures. However, continuous, serial cultures may allow for the occurrence of genetic drift and increase the likelihood of contamination (Day and DeVille 1995; Day et al. 2000). Further to this, continuous maintenance of actively growing algal strains over long periods of time is also costly. To reduce these adverse effects of long-term culturing, algal cryopreservation has more recently been adopted. Anderson (2005) believes that cryopreservation is the best method for the long-term storage of living organisms (typically at temperatures lower than −130°C) whilst also maintaining the capability of microalgae species to survive after thawing despite the fact that the process often causes cellular injuries as a result of freezing induced stress. Although cryopreservation is still empirical and its underlying mechanism of cell injury in the process of freezing and thawing is not fully appreciated (Baust 2002), a decent number of cyanobacteria and eukaryotic microalgae have been successfully cryopreserved. Cryopreservation is established as the best method for preservation at major algal collections, including the American Type Culture Collection (Lee and Soldo 1992), Culture Collection of Algae and Protozoa (Morris 1978), the Provasoli Guillard National Center for Culture of Marine phytoplankton (Andersen 2005), Pasteur Culture Collection and the Culture Collection of Algae at University of Texas-Austin (Bodas et al. 1995).

Here, we report the result of cryopreservation of psychrophilic microalgae with a biotechnological potential as a means of maintaining their genetic integrity.

2. Materials and methods

Strain and culture condition

Porosira sp. and *Pyramimonas* sp. were obtained from Maxwell Bay, King George Island, Antarctica (Figs. 1 and 2). Strains were isolated by sterile Pasteur pipettes, washed, and grown in modified f/2 medium. They were cultured at approximately 4°C under 25 μmol photons·m^{-2}·s^{-1} (24 h light) at the Korea Polar Research Institute.

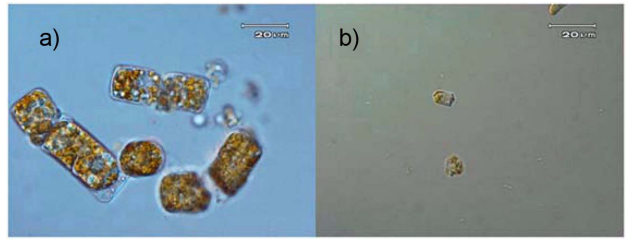

Fig. 1. Optical microscopy of *Porosira* sp. and *Pyramimonas* sp. (Scale bars: 20 μm; a, *Porosira* sp.; b, *Pyramimonas* sp.)

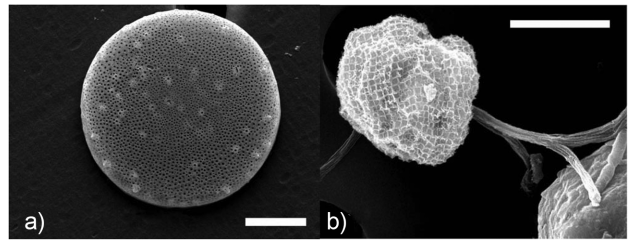

Fig. 2. Scanning electron microscopy (SEM) of *Porosira* sp. and *Pyramimonas* sp. (Scale bars: 5 μm; a, *Porosira* sp.; b, *Pyramimonas* sp.)

Measurement of antifreeze activity

Antifreeze activity was measured using a nanoliter osmometer (Otago Osmometers, Dunedin, New Zealand) connected to a cold well stage mounted on an Olympus model CH-2 microscope stage equipped with a Canon Digital Camera. Briefly, about 2 ml of supernatant of microalgae culture media was loaded into the nanoliter osmometer well and frozen rapidly below −20°C. The ice was warmed up slowly until only single ice crystal remained. The temperature was lowered again continuously at a cooling rate of 0.05°C/min. Ice crystal morphology changes were also observed and recorded as temperature decreased. When solution has antifreeze activity, the ice seed starts to shape into hexagonal ice and maintains its shape until the solution reaches a non-equilibrium freezing point. As temperature goes lower than the non-equilibrium freezing point, the hexagonal ice crystal bursts. On the contrary, when there is no antifreeze activity in solution, the ice seed starts to grow amorphously. The temperature difference between non-equilibrium freezing point and melting point caused by antifreeze activity is called thermal hysteresis (TH).

CPA toxicity test

To determine the toxicity of various cryoprotectants (CPAs) to the microalgae strains, the following two

variables were evaluated: Kinds of CPAs (dimethyl sulphoxide, DMSO; ethylene glycol, EG; glycerol, Gly; methanol, MeOH; and propylene glycol, PG), and their concentrations (10, 20, 30, 40%; v/v). All chemicals were reagent-grade and CPA solutions were prepared in autoclaved seawater.

About 1.4×10^6 cells/ml of *Pyramimonas* sp. and 3.4×10^4 cells/ml of *Porosira* sp. were harvested using centrifugation at 3,200×g for 5 min at 4°C. Cells were then resuspended in one ml of each of the five CPAs at the four different concentrations, incubated for 10 and 30 min at 4°C, washed with autoclaved seawater, and centrifuged at 3,200 g for 5 min. The resulting pellet was suspended in 35 ml of f/2 medium. Cultures were grown as outlined above for 20 days. The number of cells was determined in diluted aliquots by counting in a Thoma hemocytometer under a microscope. Each measurement was done in triplicate.

Cryopreservation

Cryopreservation was employed by (a) direct freezing in liquid nitrogen and following storage at the same temperature, and (b) modified freezing in a controlled rate freezer (Kryo 560, Planner, UK) before storage in the liquid nitrogen (LN_2) tank (VHC 35, Tailor Wharton, USA). Direct freezing was performed by immersing the cryogenic tube containing CPA treated cells into LN_2 tank. Modified freezing protocol was adopted from Andersen (2005). Cryogenic vials (Cryotube 1.8 ml, Nunc, Denmark) containing one ml of CPA-treated cells (as described above) were cooled from 4°C to –9°C at a rate of -1°C min^{-1}. As a result seawater remained supercooled at this temperature. The cooling chamber was then cooled rapidly to –45°C in order to drive the contents of cryogenic vials down to –12°C. This step caused the formation of ice nucleation and subsequent release of harmful latent heat. To remove the latent heat, the temperature of the chamber was raised quickly up to –25°C. The contents of vials were then cooled again at -1°C min^{-1} until –45°C, which was below the eutectic temperature. Lastly, the vials were then cooled rapidly down to –90°C and transferred from the cooling chamber to a liquid nitrogen tank. After freezing, the samples were preserved in liquid nitrogen tank for 7 days. Rapid thawing for both freezing methods was performed by immersing the cryogenic vials in a 37°C water bath for 2 min 30 sec. The cryogenic vials were then centrifuged at 10,000 rpm, at 4°C for 2 min and CPAs were discarded. The algal pellets were resuspended in 35 ml of f/2 medium at 4°C. Cultures were grown for 20 days, and viability assays were conducted every 3 days. The most reliable method to measure cell viability is to count the cells in every sample. Viable cells were counted using a Thoma hemocytometer under a microscope. Each measurement was done in triplicate.

3. Results

CPAs toxicity test

As shown in Table 1 and 2, all CPAs at over 30% concentration were observed to be toxic to both strains regardless of equilibration time. Of the five CPAs tested, DMSO and PG showed the least toxicity to both strains, while the other three CPAs were tolerable only up to a concentration level of 10%. *Porosira* sp. and *Pyramimonas* sp. showed similar susceptibility to CPAs. However, *Pyramimonas* sp. tolerated each of the CPAs at a higher

Table 1. Effects of various CPAs at different concentrations on *Porosira* sp. (KOPRI AnM0008)*

CPA	Concentration (%)	Equilibration time (min)	
		10	30
Dimethyl sulphoxide (DMSO)	10	+2.1	+1.6
	20	+1.8	+0.4
	30	-	-
	40	-	-
Ethylen glycol (EG)	10	+1.8	+1.9
	20	-	-
	30	-	-
	40	-	-
Glycerol (Gly)	10	+4.1	+3.4
	20	-	-
	30	-	-
	40	-	-
Methanol (MeOH)	10	+1.5	+1.4
	20	-	-
	30	-	-
	40	-	-
Propylene glycol (PG)	10	+1.9	+2.7
	20	+1.3	+1.8
	30	-	-
	40	-	-

**Porosira* sp. was incubated at 4°C for 10 and 30 min with various concentrations (10, 20, 30, and 40%) of CPAs. Effects on viability are indicated by culture color and cell concentration$\times10^4$ cell ml^{-1}. Liquid cultures with no green color after 20 days of illumination were indicated as "–"; and those which had grown to a dense brown color as "+"

Table 2. Effects of various CPAs at different concentrations on *Pyramimonas* sp. (KOPRI AnM0046)*

CPA	Concentration (%)	Equilibration time (min)	
		10	30
Dimethyl sulphoxide (DMSO)	10	+3.3	+0.6
	20	+1.8	+0.3
	30	-	-
	40	-	-
Ethylen glycol (EG)	10	+1.5	+0.1
	20	+0.7	-
	30	-	-
	40	-	-
Glycerol (Gly)	10	+0.6	+0.4
	20	+0.1	-
	30	-	-
	40	-	-
Methanol (MeOH)	10	+1.3	+0.3
	20	+0.4	-
	30	-	-
	40	-	-
Propylene glycol (PG)	10	+0.9	+0.6
	20	+0.3	+0.1
	30	-	-
	40	-	-

**Pyramimonas* sp. was incubated at 4°C for 10 and 30 min with various concentrations (10, 20, 30, and 40%) of CPAs. Effects on viability are indicated by culture color and cell concentration$\times 10^6$ cell ml^{-1}. Liquid cultures with no green color after 20 days of illumination were indicated as "–"; and those which had grown to a dense green color as "+"

concentration level of 20% for an equilibrium time of 10 min.

Cryopreservation

Based on CPA toxicity test, CPAs with a concentration of 10% and 20% were treated for a 10 min period only. Of the two strains cryopreserved, only *Pyramimonas* sp., a psychrophilic chlorophyta was revived following both freezing methods. On the other hand, *Porosira* sp. was unable to be revived following either of the two freezing methods (Table 3). At 10% and 20% concentration of DMSO, all cells using both direct and modified freezing methods were revived. There was no revival from direct freezing at 10% or 20% of EG, 10% or 20% of Gly, and 20% of MeOH. However, all cells were revived at all concentrations of the five CPAs following the modified freezing method (Table 3).

Table 3. Result of Cryopreservation of *Pyramimonas* sp. (KOPRI AnM0046)*

CPA	Concentration (%)	Cryopreservation protocol	
		DF	MF
Dimethyl sulphoxide (DMSO)	10	+	+
	20	+	+
Ethylen glycol (EG)	10	-	+
	20	-	+
Glycerol (Gly)	10	-	+
	20	-	+
Methanol (MeOH)	10	+	+
	20	-	+
Propylene glycol (PG)	10	+	+
	20	+	+

*After cryopreservation, *Pyramimonas* sp. was incubated at 4°C for 20 days. Liquid cultures with no green color after 20 days of illumination were indicated as "–"; and those which had grown to a dense green color as "+". DF = direct freezing, MF = modified freezing

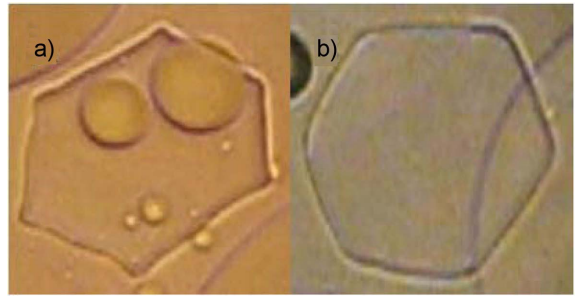

Fig. 3. Antifreeze activity by culture medium of *Pyramimonas* sp. before (a) and after (b) cryopreservation

Measurement of antifreeze activity

Antifreeze activity of *Pyramimonas* sp. was tested before and after thawing from cryopreservation (Fig. 3). As shown in Fig. 3, the antifreeze activity, demonstrated as shaping the hexagonal ice crystal, was maintained after thawing the cryopreserved cells, meaning the cells were cryopreserved intact to a certain degree. Thermal hysteresis caused by the cell was 0.5°C before and after cryopreservation of *Pyramimonas* sp.

4. Discussion

In response to current demands in biology, preservation methods for many types of cells and organisms are under development. Cryopreservation is used to sustain genetic stability and cell integrity during long periods of

preservation and successful cryopreservation of many organisms and cells have been achieved in liquid nitrogen (Bajaj and Reinert 1977; Leeson et al. 1984; Rall and Fahy 1985; Mazur et al. 1992). However, the cryopreservation of marine algae has not been established well (Terumoto 1965; Leeson et al. 1984; van der Meer and Simpson 1984; Fenwick and Day 1992; Kuwano et al. 1992, 1993; Fujiyoshi et al. 1993).

The addition of cryoprotective agents prior to freezing is critical for cryopreservation. Cryoprotectant's toxicity is well documented (Fahy 1986), and maximum tolerable concentrations are used in order to achieve the highest viability and to be less dependent on cooling and warming rates during the freezing and thawing processes (Morris and Farrant 1972). As a result of CPA toxicity test, *Porosira* sp. and *Pyramimonas* sp. were found not to survive at the concentrations over 30% of any CPAs tested. Therefore, optimum concentrations and equilibration time for the five CPAs were determined to be 20% and 10 min, respectively.

The aim of this study was to cryopreserve polar microalgae and to research optimal cryopreservation conditions. As a result of this study, cryopreservation of *Pyramimonas* sp. was found to be successful using either direct freezing or modified freezing methods, both, without reducing antifreeze activity. The cryopreservation of *Porosira* sp., on the other hand was not met with such success. This may be due to a change in the characteristic properties of the cell wall. During the process of freezing and thawing diatoms several times, their silica cell wall may suffer extensive shrinkage, which makes cryopreservation of diatoms unfavorable. Another explanation may be related with size of *Porosira* sp.. The size of the cell is large (approximately 20 mm in diameter), which often causes a problem during freezing. Large cells with a low surface area/volume ratio loses water less effectively during freezing than smaller cells with high surface area/volume ratios, making them prone to inner ice formation during freezing (Grout and Morris 1987).

Various cell types including green algae have been cryopreserved for years with no decrease in viability during storage at low temperatures (Doebbler et al. 1966; Hwang and Hudock 1971; Day et al. 1997) as cryobiologists believe that cells may be stored indefinitely in liquid nitrogen without loss of viability (Mazur 1984). Based on our results and other reports, it should be feasible to cryopreserve some polar microalgae from KCCPM for a long-term, using the freezing protocol shown in this report.

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