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Optimization of cryopreservation conditions for the unicellular red alga *Cyanidioschyzon merolae*

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Cryopreservation is essential for maintaining stable stocks of organisms. We report the development of a method for cryopreservation of the unicellular red alga *Cyanidioschyzon merolae*, a model organism for the investigation of the basic architecture of photosynthetic eukaryotes. Glycerol, dimethyl sulfoxide and methanol were examined for their ability to protect the cell from cryoinjury and/or cytotoxicity. It was found that methanol was the most effective as a cryoprotectant for *C. merolae*. After the optimized setting of parameters such as working concentration of cryoprotectant and the period of slow cooling, cultures were supplemented with 5% (v/v) methanol and frozen by slow cooling using a passive-freezing unit, followed by plunging into liquid nitrogen. We found *C. merolae* cells retained greater than 80% viability for at least 83 days in storage.

Key Words——cryoinjury; cryopreservation; *Cyanidioschyzon merolae*; unicellular red alga

Introduction

Cryopreservation is a method for long-term storage of living cells or tissues at ultra-low temperatures, such as those experienced in liquid nitrogen. Cryopreservation enables the storage of rare organisms and those that are difficult to stably maintain in culture. Additionally, cryopreservation of type strains is very important to maintain a supply of genetically stable stock.

Since microalgae have recently gained attention as a useful organism in applications such as bio-energy production (Li et al., 2008; Rittmann, 2008) and bioremediation (Perales-Vela et al., 2006), the nucleotide sequences of several algal genomes have been determined (Armbrust et al., 2004; Derelle et al., 2006; Matsuzaki et al., 2004; Merchant et al., 2007; Nozaki et al., 2007; Palenik et al., 2007) and the sequencing of others is ongoing (Grossman, 2005, 2007; Parker et al., 2008). Cryopreservation of these algae has been becoming increasingly important to preserve this genomic information.

Cyanidioschyzon merolae, a unicellular red alga, has a simple cell structure, one nucleus, one mitochondrion, one plastid and a minimal set of membrane-bound organelles: one microbody (peroxisome), one Golgi apparatus and a small number of vacuoles (Kuroiwa et al., 1994). Phylogenetic analyses have demonstrated that *C. merolae* diverged very early in the eukaryotic lineage (Nozaki et al., 2003), and it is thought that *C. merolae* retains primitive but essential cellular functions. The complete sequence of the *C. merolae* genome was first determined (Matsuzaki et al., 2004; Nozaki et al., 2007; Ohta et al., 1998, 2003). Genomic analyses have revealed that *C. merolae* has

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the simplest genome with the smallest redundancy of any photosynthetic eukaryote analyzed to date (Matsuzaki et al., 2004; Nozaki et al., 2007). Taking advantage of genomic information and transformation techniques (Minoda et al., 2004; Ohnuma et al., 2008), we disrupted certain genes by homologous recombination (Imamura et al., 2009, 2010).

To preserve wild-type and mutant strains, development of a cryopreservation method for *C. merolae* is essential. However, *C. merolae* is highly sensitive to mechanical injury because of the absence of a cell wall (Kuroiwa et al., 1994), and thus the usual freezing process is deleterious to cell viability (our unpublished observations). In this study, we have developed a successful cryopreservation method for *C. merolae*.

Materials and Methods

Algal strain and culture conditions. *C. merolae* 10D, which is available at The Microbial Culture Collection at the National Institute for Environmental Studies, Tsukuba, Japan (NIES-Collection, collection number: NIES-1332), was used in this study. *C. merolae* cells were grown in MA2 medium (Ohnuma et al., 2008) in a glass vessel under continuous white light (50 μmol photon m^{-2} s^{-1}) at 40°C with bubbling 2% CO_2.

Cryopreservation procedure. Stationary phase cultures, defined as those where the absorbance at 750 nm [A_{750}, using spectrophotometer, DU 600 (Beckman Coulter, Brea, CA, USA)] was greater than 10, were mixed with a cryoprotectant solution such as glycerol, dimethyl sulfoxide (DMSO) or methanol to obtain a concentration of 6.5×10^8 cells ml^{-1}, and 500 μl aliquots were dispensed into 1.5 ml tubes. Samples were placed in a Mr. Frosty passive freezing unit (Nalge Nunc International, Rochester, NY, USA) at room temperature, then placed in a −80°C freezer for 3 h. Samples were removed from the Mr. Frosty and immediately transferred into liquid nitrogen for long-term storage.

Assessment of cell viability and growth after thawing. For thawing, samples were removed from liquid nitrogen storage and placed in a water bath at 42°C in the dark. Immediately after thawing, 100 μl of the sample was diluted in 1 ml MA2 medium and incubated overnight at 40°C/5% CO_2, in the dark. Viability was then assessed by staining with fluorescein diacetate (FDA; Sigma, St. Louis, MO, USA) as described previously (Mori et al., 2002). The FDA-stained cells were counted using a microscope, and the survival rate was calculated. More than 300 cells from at least three different fields of microscope were counted for each sample. The survival rate after incubation for 3 h at room temperature without cryoprotectant was set at 100%. Experiments were repeated three times to examine reproducibility.

To examine growth after freezing for various periods of time, 250 μl aliquots of thawed cells were diluted with 1 ml MA2 medium in 24-well microtiter plates. The samples were serially diluted 10-fold to 10^{-7}. Plates were incubated at 40°C/5% CO_2, in the dark for 1 day and then under continuous white light. Plates were then examined for cell growth. Experiments were performed in triplicate, and cell viability was also assessed also by the most probable number (MPN) method (Fenwick and Day, 1992). The MPN value was calculated using the MPN Calculator (http://www.i2workout.com/mcuriale/mpn/index.html) using cultures after 12 days of growth.

Results and Discussion

Freezing of algal cells usually damages cells and results in severe loss of viability; however, several cryopreservation methods have been developed. Slow cooling prior to immersion into liquid nitrogen is usually effective at increasing the viability of microalgae following cryopreservation (Crutchfield et al., 1999; Day, 2007; Day et al., 2008; Taylor and Fletcher, 1999). For *Chlamydomonas*, a final culture density of 3.3×10^6 cells ml^{-1} was mixed with methanol (2–10%) in a cryovial and the cryovial was placed in a pre-chilled (4°C) Mr. Frosty, a commercially available passive freezing unit (Crutchfield et al., 1999). After passive freezing in a −70°C freezer for 1.5 h, cryovials were removed from Mr. Frosty and immediately plunged into liquid nitrogen. In this study, we examined choice and working concentration of cryoprotectant, the period of slow cooling and effects of cell density, using Mr. Frosty. We also analyzed cell viability after extended cryopreservation periods.

We employed FDA-staining, which is a commonly used method to assess viability of microalgae (Day and DeVille, 1995; Fleck et al., 2000; Mori et al., 2002). When FDA permeates a cell, it is hydrolyzed to produce fluorescein (Rotman and Papermaster, 1966) and viable cells exhibit a strong fluorescent signal.

We tested some commonly used cryoprotectants to

find suitable agents for *C. merolae*. Cells were diluted with MA2 medium containing 5 or 10% glycerol, DMSO or methanol to achieve a concentration of 6.5×10^8 cells ml^{-1}, and were incubated at room temperature or subjected to cryopreservation treatment. As shown in Fig. 1A (upper panel), when cells were incubated at room temperature for 3 h, the survival rate was greater than 86%, regardless of the cryoprotective agents. Among the agents tested, higher survival rates were obtained when the cells were frozen with 5 or 10% DMSO or 5% methanol. Glycerol was not effective in protecting against cryoinjury of *C. merolae.* No cryoprotectant tested was effective for survival when the cells were rapidly frozen (data not shown). As shown in Fig. 1A (lower panel), cells treated with 5% methanol yielded the best results. We examined the effects of DMSO and methanol concentration on survival rate. A survival rate greater than 50% was observed when cells were frozen with DMSO in the range of 5−10% or with methanol in the range 4−7% (Fig. 1B). The cells frozen with 5% methanol grew normally while cells frozen with 7.5% DMSO did not (Table 1). Thus, DMSO appears to be effective as a cryoprotectant but harmful to cell growth. Although the range of optimum concentration of methanol was narrower than for DMSO, methanol had less adverse effects on cell growth (Fig. 1A, Table 1). We concluded that MA2 medium supplemented with 5% methanol was suitable for cryopreservation of *C. merolae*, and therefore used 5% methanol as the cryoprotective agent in subsequent experiments.

We examined the effects of duration of the initial slow cooling and cell density on the survival rate to determine optimum conditions for cryopreservation. Cells were diluted with MA2 medium containing 5% methanol, placed into Mr. Frosty and transferred to a −80°C freezer. After incubation for 1, 2, or 3 h or overnight, samples were removed from Mr. Frosty and im-

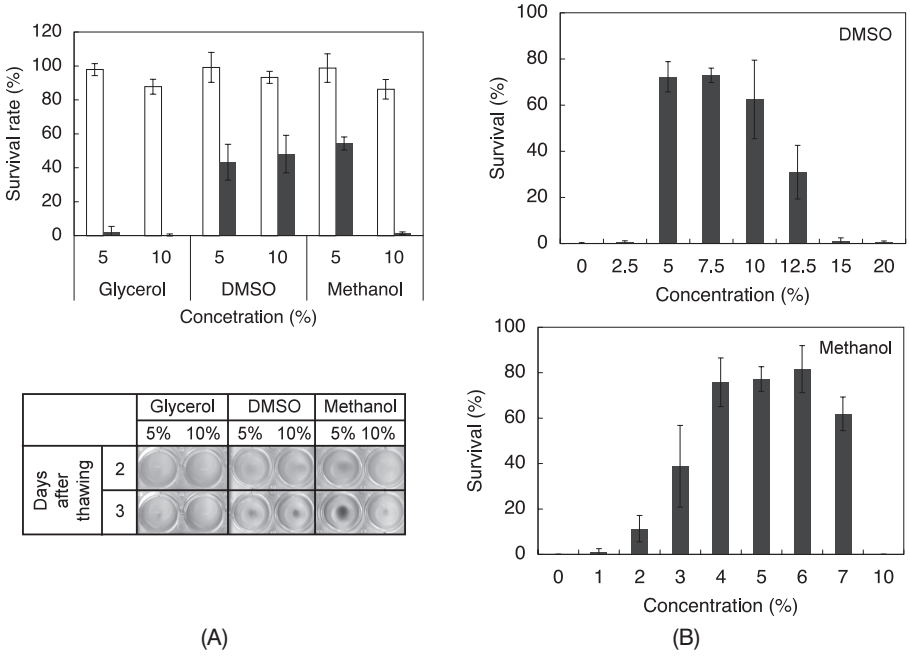

Fig. 1. Selection of cryoprotective agents for *C. merolae*.

(A) Survival rates after cryopreservation treatments with various cryoprotectants (upper panel). Cell suspensions were supplemented with the indicated cryoprotectants. Survival rate after immersing in liquid nitrogen following slow cooling for 3 h (filled column) or after incubating for 3 h at room temperature (unshaded column) were examined. Cell cultures after thawing are shown in the lower panel. Thawed samples were diluted and incubated at 40°C overnight in the dark and then incubated under continuous illumination. (B) Optimum concentrations of DMSO and methanol. Cells were diluted with MA2 medium containing the indicated final concentration of DMSO (upper panel) or methanol (lower panel) and subjected to cryopreservation. The survival rate after immersion in liquid nitrogen was examined.

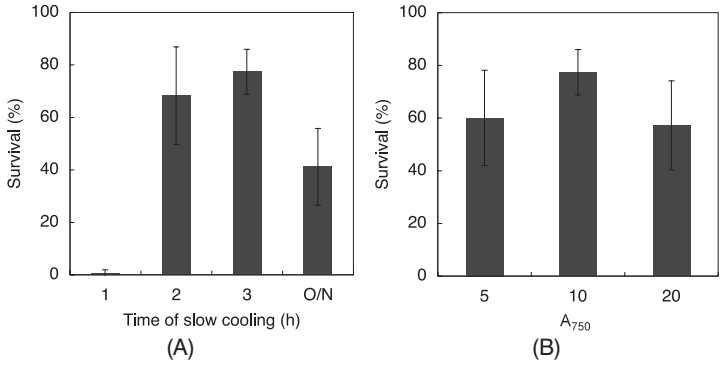

Fig. 2. Optimum conditions of cryopreservation.

Survival rates of thawed samples after immersion in liquid nitrogen were examined. (A) Period of slow cooling. Samples were plunged into liquid nitrogen after the indicated period of slow cooling. O/N, overnight. (B) Concentration of cell cultures. Cell cultures were diluted or concentrated, and mixed with MA2 medium containing methanol [(final concentration (5%)] to adjust the A_{750} to 5, 10 or 20.

Table 1. Effects of cryoprotectants on cell proliferation of *C. merolae*.

Cryoprotectant	A_{750}	
	Control	Frozen
7.5% DMSO	6.52	0.69
5% methanol	7.13	5.04

Cells mixed with the indicated cryoprotectants were either immersed in liquid nitrogen after slow cooling for 3 h (Frozen) or following incubation for 3 h at room temperature (Control). Immediately after thawing, samples were diluted and cultured as described in MATERIALS AND METHODS. Cell proliferation was assessed by measuring the optical density (A_{750}) after 7 days of growth.

mediately transferred into liquid nitrogen. As shown in Fig. 2A, an initial slow cooling period for 1 h was not sufficient. A higher survival rate was obtained when slow cooling was conducted for 2 or 3 h, while overnight slow cooling decreased survival rate. With respect to effects of cell density on the survival rate, cell cultures at A_{750} = 14.1 were diluted or concentrated and supplemented with methanol [final concentration 5% (v/v)] to achieve an A_{750} of 5, 10 or 20 (approximately 3.2×10^8, 6.5×10^8 and 13.1×10^8 cells ml^{-1}, respectively). When the cell density was in the range of A_{750} = 5–20, the survival rate was not notably different. A cell density of A_{750} = 10 gave the most stable result (Fig. 2B).

C. merolae cells were cryopreserved using the determined optimized conditions and at least 60% of the cells were found to have survived after storage at every examined time point (Fig. 3A). When the cells were stored in liquid nitrogen for longer, cell growth and MPN values gradually decreased (Fig. 3B, bottom column). Although we did not check prolonged periods of preservation, we observed cell growth after freezing treatment using a self-produced box that is a plastic 1.5 ml tube rack fixed in container made of Styrofoam (data not shown).

While we were able to obtain satisfactory viability using our protocol, cell proliferation was gradually reduced during prolonged periods of preservation. Further improvement is likely required to prevent the gradual reduction of cell proliferation for longer-term storage. However, this problem could be temporarily solved by increasing the initial number of cells. The demand for cryopreservation of microalgae will increase with developments in the field of algal genomics. Our methodologies may also be applicable to other species that lack cell walls, such as *Dunaliella* (Eyden, 1975), and contribute to microalgal culture collections in laboratories or biological resource centers.

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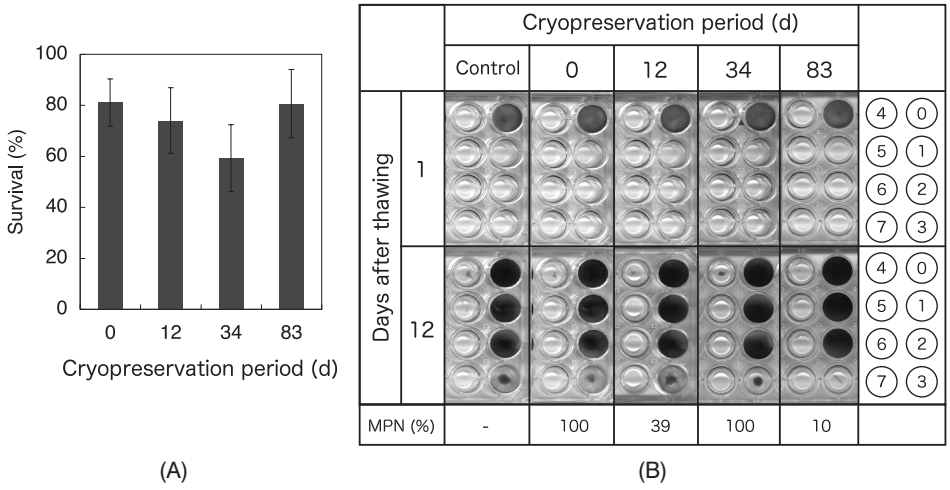

Fig. 3. Effect of cryopreservation period.

(A) Survival rates of samples that were thawed after the indicated periods of cryopreservation. (B) Effects of cryopreservation period on cell proliferation. Typical culture among three thawed samples is shown. Cell suspensions that were supplemented with methanol [final concentration (5%)] and incubated at room temperature for 3 h are shown as a control. The 250 μl aliquot of thawed samples were diluted in 1 ml of MA2 medium. Then samples were serially diluted 10-fold to 10^{-7}. The number of the circle in the right column corresponds to the exponent of the dilution factor. The calculated MPN value is indicated as a percentage of the control (bottom column).

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