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Procedia Environmental Sciences 15 (2012) 118 – 125

Procedia Environmental Sciences

The 1st Asia-Oceania Algae Innovation Summit (AOAIS-1)

Cryopreservation of the chlorophyll *d*-containing cyanobacterium *Acaryochloris marina*

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Abstract

Cryopreservation of a prokaryotic alga, *Acaryochloris marina*, which was first isolated as a minor symbiont from a colonial ascidian, was investigated. This alga, possessing chlorophyll *d* as the major photosynthetic pigment, is an important organism for elucidating various biological questions such as the mechanism of chlorophyll diversity and the evolution of photosynthesis. However, the proportion of photosynthetic pigments changes on serial subcultures on maintenance under a fluorescent lamp, a light condition that differs from that of the original habitat. Cryopreservation has the capacity to ensure the maintenance of the physiological and genetic stability of *A. marina*. By placing samples in a styrene foam container in a deep freezer, cooling rate of –2°C·min^{-1} was obtained for pre-freezing to –80°C prior to plunging into liquid nitrogen. Amongst the three distinct cryoprotectants employed, [dimethyl sulfoxide (DMSO, 10% v/v), methanol (10% v/v), and glycerol (10% v/v)], DMSO was the most effective since cells preserved in DMSO for one month in liquid nitrogen or at –80°C in a deep freezer, started to grow 200 h after inoculation at 20°C under a fluorescent lamp . In contrast, no cell growth was observed when cells were preserved employing methanol, and growth of contaminant bacteria was observed when cells were preserved in glycerol. The optimum concentration of DMSO was 10%. Other concentrations of DMSO resulted in an extended lag period at 5% and 20% DMSO, and growth was completely inhibited at 40% DMSO.

Keywords: Acaryochloris marina; cryopreservation; dimethylsulfoxide; Styrofoam box

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doi:10.1016/j.proenv.2012.05.016

1. Introduction

The prokaryotic photosynthetic organism *Acaryochloris marina* is an alga classified as a Cyanophyta. This alga possesses unique physiological properties, containing chlorophyll *d* as the dominant light-absorbing pigment instead of chlorophyll *a* [1]. The habitat of the alga is considered to be global because chlorophyll *d* has been detected in various locations [2] and in sedimentary samples [3]. Futhermore *Acaryochloris* sp. (AWAJI-1) was isolated from epiphytic patches on the thallus of red algae [4]. The first strain of this genus isolated, *A. marina* MBIC11017, was obtained in 1996 as a symbiont in colonial ascidians from the tropical coast of the Republic of Palau [1]. Originally, this strain did not have a large quantity of phycobiliproteins; however, the pigment profile changed over time in culture over an extended period under cool white fluorescent lamps and the profile has not return to original state up to the present [5]. Therefore, it is essential to develop a method for maintaining the strain without genetic and physiological changes. However, stable, long-term preservation methods for *Acaryochloris* have never been established so far.

Although serial subculture does not require special equipment and is a commonly used process, it is labor intensive and has many opportunities for contamination. The possibility of genetic drift is another disadvantage of the method because organisms may adapt to the laboratory environment, and hence, the culture may "evolve" to favor laboratory conditions as it undergoes thousands of subculture transfer. A variety of methods have been applied to maintain stock cultures of algae in stable state for a long time, such as liquid drying (L-drying), freeze-drying, and storage at low and ultra-low temperatures. The freeze-drying technique has been used for some algae but has been unsuccessful for most algae [6]. Cryopreservation has been applied for the preservation of various algae such as cyanobacteria, green algae, red algae, brown algae, euglena, diatoms, and coccolthophorids [7, 8, 9, 10, 11, 12]. However, to ensure successful preservation of an individual algal strain the rate of freezing, the type of cryoprotectants, the concentration of cryoprotectants, and the conditions of warming require optimization [13, 14]. The present study details the development of a cryopreservation technique for *Acaryochloris*.

2. Materials and methods

2.1 Algal material and culture

Acaryochloris sp. MBIC10697 containing chlorophyll *d*, was grown in natural seawater enriched with Daigo's IMK medium (NS-IMK medium) (Wako, Osaka, Japan). For stock cultures, cells were maintained in 100-mL Erlenmeyer flasks containing 50 mL of medium under a 16-h light/8-h dark regime. The light intensity determined by a photon sensor (IKS-25; Koito Manufacturing Co., Ltd, Tokyo, Japan) at the surface of the vessel was 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Growth tests were performed in an L-shaped tube (Taitec, Tokyo, Japan) under continuous high light intensity (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 20°C.

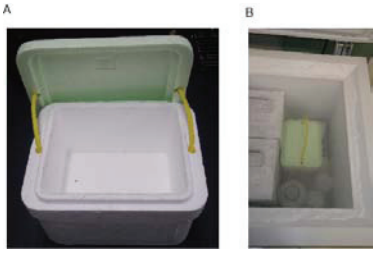

Fig. 1. A styrofoam box used in this study (A) and the setting in a -80 ºC deep freezer MDF-192 (B).

2.2 Cryopreservation

Aliquots (1 mL) of algal culture in late log-phase growth was cooled to –40°C in 2 mL sample tubes in a –80°C deep freezer (MDF-192; Sanyo, Osaka, Japan) for one hour. In this step, sample tubes were placed directly in the freezer or indirectly with wrapping in a bubble pack sheet inside a Styrofoam box (Figure 1). The change in temperature in the first freezing was monitored using a thermo recorder (RT-12; Espec Mic, Aichi, Japan). Cell suspensions were then cooled rapidly to –196°C or –80°C by immersing them in liquid nitrogen or by directly placing them in the deep freezer. To test the efficacy of 3 cryoprotectants, dimethyl sulfoxide (DMSO), methanol, or glycerol, were added to the cell suspensions before freezing to a final concentration of 10% (v/v). The cell suspensions were thawed by rapid agitation of the tube in a water bath at 37°C until the ice crystal melted. The thawed suspensions were then transferred to 50 mL sample tubes and diluted on ice with fresh NS-IMK as detailed in Table 1 with each step lasting 1-min.

Table 1. Dilution procedure after thawing.

Step	Fresh medium added (mL)	Total volume (mL)	Concentration of cryoprotectant* (%)
0 (after thawing)	0	1	10
1	0.5	1.5	6.7
2	0.5	2	5
3	1	3	3.3
4	2	5	2
5	5	10	1
6	10	20	0.5
7	10	30	0.3

*The initial concentration was 10% (v/v) cryoprotectant in the cell suspension.

2.3 Growth assay

Ten milliliters of the algal suspension was transferred to an L-shaped tube and incubated as described above for the growth assay. Changes in cell concentrations during cultivation were monitored by measuring the optical density using a spectrophotometer (Spectronic 20A, Shimadzu, Kyoto, Japan) at 750 nm (expressed as OD_{750}).

3. Results and Discussion

3.1. Freezing and warming

Successful cryopreservation is dependent on the optimization of a number of parameters including final storage temperature. Although it is possible to preserve some cyanophycean algae at –80°C [15], for most successfully cryopreserved algal strains storage at –196°C using liquid nitrogen or vapor-phase nitrogen is optimal, because viability levels decreased when samples were stored at temperatures above –70°C [16]. The cooling rate is also a critical factor for achieving a frozen-hydrated state by vitrification [13, 14]. States of intra- and extracellular water and survival rates during cryopreservation were described by Mazur [17]. In the vitrified state, water molecules are solidified into a glass-like structure without forming ice crystals. Because ice crystals occupy greater cell volume and cause pressure and shearing

forces on intracellular organelles, cells are damaged and the survival rate is reduced. If cooling is too rapid, excess intracellular water is not sufficiently dehydrated and becomes supercooled. As a result, the possibility of cell damage increases by ice crystal formation in the cell. On the other hand, if cooling is too slow, cells loose the intracellular liquid by excessive dehydration and are subject to chemical and physical damage such as that by increase in toxicity of intracellular solutes and crushing of intracellular organelles. Therefore, a 2-step controlled cooling method, which consists of an initial slow cooling step and a subsequent step of rapid cooling to the temperature of liquid nitrogen (Fig. 2A) has been widely applied in the cryopreservation of algal strains [7, 14, 18]. In general, the initial cooling is performed at the rate of approximately –1°C·min^{-1} to a holding temperature of around –40°C [13, 14]. Using a program freezer, it is possible to control the final temperature and cooling rate accurately, however, the equipment is expensive, and not all laboratories can afford it. Therefore, some laboratories use proprietary passive coolers such as Cryo 1°C Freezing Container, "Mr. Frosty" (Nalge Nunc International Corporation, NY, USA). In this study the 2 mL-sample tubes were wrapped with bubble wrap, placed in a foamed polystyrene box, and then the box was placed in a –80°C deep freezer (Fig. 1). Subsequently, the sample tubes were rapidly cooled using liquid nitrogen, or by placing in a deep freezer. Fig. 3 shows the temperature changes of the sample tube filled with distilled water in the first step. When the tube was directly placed in the freezer, the temperature dropped at a rate of approximately –4°C·min^{-1} (Fig. 3). In the case of indirect freezing, the rate was –2°C·min^{-1}. Since a temperature plateau was observed at –10°C, the temperature rate was –1°C·min^{-1} on an average i.e. the standard cooling-rate routinely employed for algae [13, 14]. Thawing is also an important step for successful cryopreservation [19]. In general samples must be warmed as fast as possible to prevent the formation and growth of ice crystals within the cells, which invariably result in damage to ultrastructual architecture and cell death (Fig. 2B) [11, 17]. High concentrations of cryoprotectant may be toxic to sensitive taxa, a gradual dilution is required to prevent drastic changes of osmotic pressure by the addition of fresh medium for subsequent cultures [11]. Therefore, the authors followed the steps shown in Table 1 for the thawing/recovery procedure.

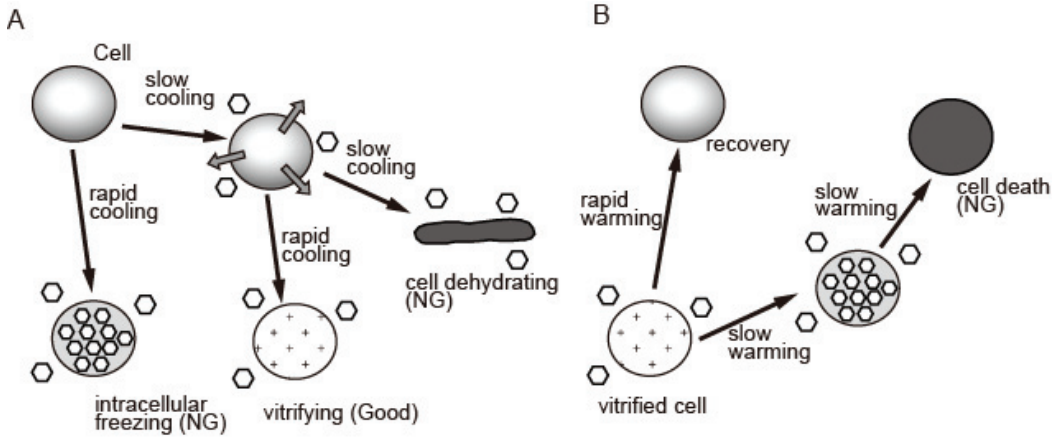

Fig. 2. Schematic representation of the physical events in cells during freezing (A) and warming (B). Hexagon, ice crystals. Modified Figure 1 in Kuwano and Saga [13].

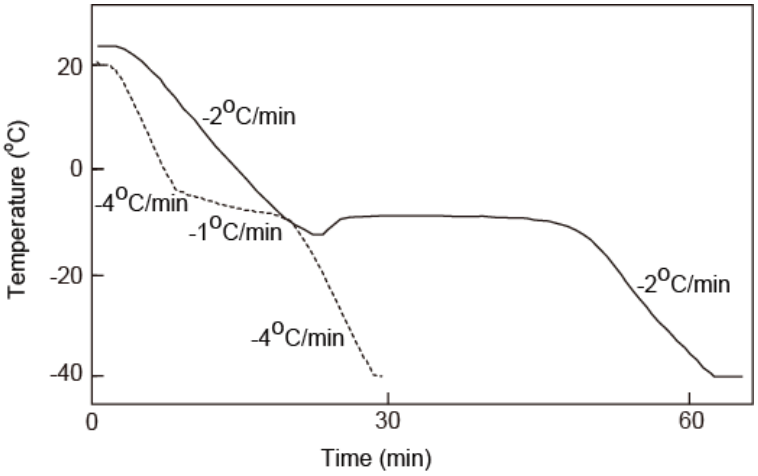

Fig. 3. Changes in sample temperatures during the first cooling period for cryopreservation.
Sample tubes were placed directly in the freezer (dashed line) or indirectly with wrapping in a bubble pack sheet within a Styrofoam box (solid line).

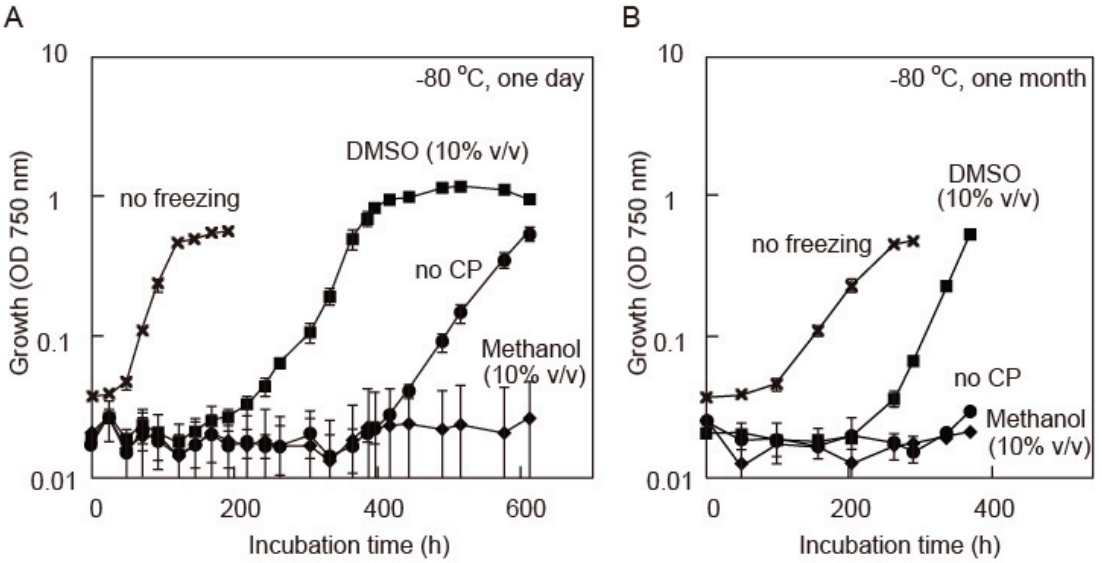

Fig. 4. Effects of cryoprotectants on the cryopreservation of *Acaryochloris*. The samples were cryopreserved at -80ºC for one day (A) or 30 days (B). Square, DMSO; diamond, methanol; circle, no cryoprotectant; X, no freezing. The final concentration in the samples was 10% (v/v).

3.2. Cryoprotectant

The cryoprotectant, sometimes called cryoprotective agent (CPA) [16], is another important factor for attaining successful cryopreservation. Various CPAs including DMSO, glycerol, and methanol have been adopted for the cryopreservation of many algae [14]. Therefore, the authors tested the protective effects of the 3 reagents at 10% (v/v) for 24-h in a –80°C freezer. After thawing, the cell suspension of *Acaryochloris*, without the addition of cryoprotectant, had a growth lag ranging from 363 to 415 h prior to growth restarting. In the samples treated with 10% (v/v) DMSO, the lag was shortened to less than half, ranging from 144 to 190 h (Fig. 4A). There is a possibility that 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ is too bright for the recovery phase and consequently the cells are damaged. This may be avoided, and the growth lag shortened by modifying the incubation conditions after thawing for example, keeping the cells in the dark for 48h and then incubating them at 10-20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ until the culture begins growing. In contrast, when ethanol was added, cells did not re-grow. In the case of glycerol, the cell suspension became clouded by the growth of contaminated bacteria, and the intended alga did not grow. The same results were obtained for samples preserved for 30 days in a –80°C deep freezer (Fig. 4B). From these results, we can conclude that cryopreservation is achievable for *A. marina*, and that DMSO was the most effective CPA employed.

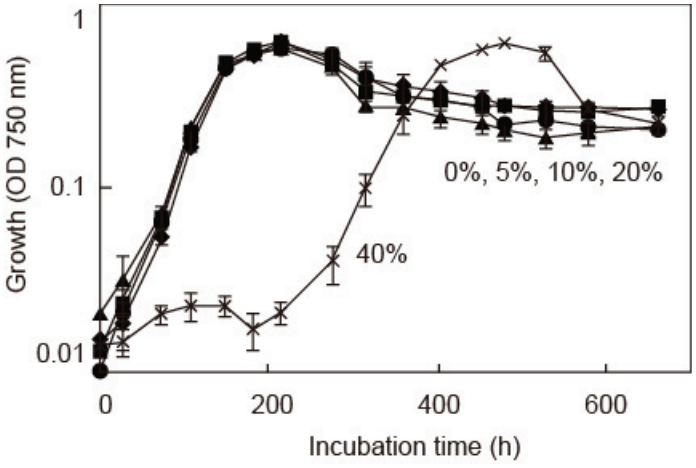

Fig. 5. Effects of DMSO on the growth of *Acaryochloris*. The final concentrations (v/v) of DMSO in the samples were 0% (circle), 5% (triangle), 10% (square), 20% (diamond), and 40% (X).

The optimum concentration and potential toxicity of DMSO were investigated. It was observed that treatment with 40% (v/v) DMSO prolonged the lag-phase to approximately 200 h, however, cells were not affected by the addition of up to 20% (v/v) DMSO under standard environmental conditions (Fig. 5). After 30 days storage in liquid nitrogen, the fastest recovery was observed in samples that had been treated with 10% (v/v) DMSO, these started to grow after approximately 190 h (Fig. 6). Longer lag-phase were observed for all other concentrations treated with lags of 210, 250, and 400h observed for thawed culture that had been treated with 5%, 20%, and 0% (v/v) DMSO, respectively. No grow was observed in samples that had been treated with 40% (v/v) DMSO (Fig. 6). These results indicated that 10% (v/v)

DMSO was optimal for cryopreservation of *Acaryochloris*. Quantitative estimation of post-thaw viability of *Mcirocystis aeruginosa* and/or the other microalgae based on the apparent increasing rate of cells [20] or FDA method [7]. The same should be investigated for *Acaryochloris* in order to get quantitative information for cryopreservation of this alga.

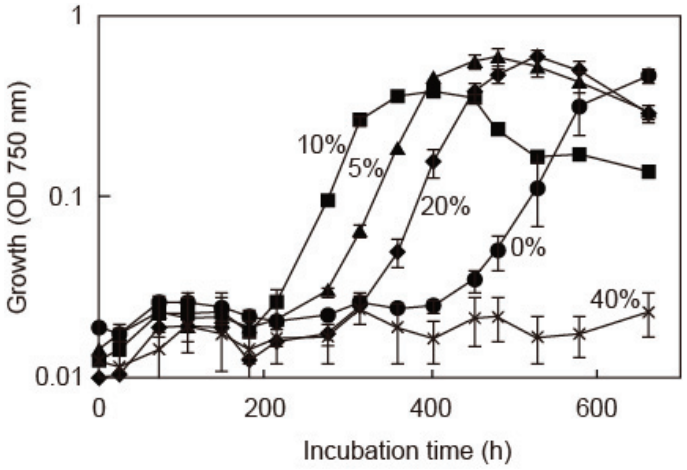

Fig. 6. Effects of DMSO on the cryopreservation of *Acaryochloris*. The samples were cryopreserved at -198ºC for 30 days. The concentrations (v/v) were 0% (circle), 5% (triangle), 10% (square), 20% (diamond) and 40% (x-indication).

4. Conclusion

The authors have demonstrated that it is possible to cryopreserve for chlorophyll *d*-containing alga *Acaryochloris*. For preservation of the alga, 10% (v/v) DMSO was the most effective cryoprotectant among those tested. Methanol completely inhibited growth, and glycerol was not suitable, because the growth of contaminant bacteria inhibited the growth of *Acaryochloris*. Accordingly this study employed a convenient, 2-step controlled cooling method, with the initial slow cooling step, using a cheap polystyrene box and bubble wrap.

Acknowledgements

We express our sincere thanks to Dr. K. Kuwano (Faculty of Fisheries, Nagasaki University) for his excellent advices and comments.

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Received 31 July 2011; accepted 30 September 2011