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Cryopreservation of Sporothalli of the Genus *Porphyra* (Bangiales, Rhodophyta) from Korea

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Cryopreservation of sporothalli of the red alga *Porphyra* (*P. seriata, P. yezoensis, P. tenera, P. pseudolinearis* and *P. dentata*) was performed by the two-step cooling method in liquid nitrogen. The algal samples were suspended in various cryoprotective solutions, and slowly cooled to -40°C in 4 hours using a programmed freezer. After the first slow cooling the suspensions with cryoprotectants were immediately immersed in liquid nitrogen. The suspension from the programmed freezer was thawed quickly by agitation of the vial in a water bath at 40°C. When ice in the suspension of cryogenic vial was mostly melted, the vial was transferred to an ice bath for complete melting of the residual ice. The cryoprotectants in the vial were washed off by gradual dilution with seawater. The viability of the cell was assessed with neutral red staining. The viability of *Porphyra* samples ranged 54.6-70.9% when the mixed suspension of 10% dimethylsulfoxide and 0.5 M sorbitol in 50% seawater used as a cryoprotectant.

Key Words: cryopreservation, *Porphyra*, sporothallus, two-step cooling method

INTRODUCTION

In response to current demands in biology, considerable effort is being directed toward the development of preservation methods for many types of cells and organisms. At present, liquid culture media have been mostly used for the preservation of algae. This liquid culture media method has advantages that are possible to culture many algae easily, but it has disadvantages of time and space consuming, possibility of pollution, deterioration of spore formation, and genetic instability when culturing for a long time. The freezing preservation method has advantages of long-term storage, a great reduction in time and space, and genetic stability, but many of the related technologies have not been developed yet (Kuwano and Saga 2000).

Many freezing methods for algae have been started with adopting land plant freezing preservation methods. In the freezing preservations of algae, 50 to 100% natural seawater are used as a freezing medium, dimethylsulfoxide (DMSO), glycerol and ethylene glycol are used for frost damage prevention materials, and sometimes proline, mannitol, dextran, sorbitol and polyvinylpyrrolidone are added in most cases. Although the application of the freezing methods may be dependent on the objects of algal samples, i.e. species, generation and age, in most cases, the two-step cooling method and the rapid thawing have been adopted in the cryopreservation. But there are still so many problems in the freezing preservation method that is suitable for samples of generation, organism, kinds of cells and physiological state; of proper freezing protocols; and of appropriate evaluation of survival and so on.

Information on cryopreservation of marine macroalgae is limited (Migita 1964, 1966, 1967; Terumoto 1965; Sakai and Sugawara 1978; van der Meer and Simpson 1984; Tsuchiya 1989, 1992, 1994; Renard *et al.* 1992; Kuwano *et al.* 1992, 1993, 1994, 1996; Fujiyoshi *et al.* 1993a, 1993b; Fujiyoshi 1997; Sakanishi and Saga 1994; Kono *et al.* 1997,

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1998), so efforts to develop the cryopreservation technique are still required because there is no universal method of cryopreservation for algae.

The modern practice in the cultivation of *Porphyra* began with free-living sporothallus cultures. These could be maintained *in vitro* by vegetative fragmentation. Vegetative fragments from stock cultures of free-living sporothalli have been used to start the large-scale culture of the sporothalli in shell substrates. The conchospores released from sporothalli could attach onto the netting twines of cultivation nets, i.e. seeding, and they have been cultivated to harvestable size in the sea. Like this, most species of *Porphyra* exhibit a biphasic life cycle in which a gametothallus alternates with a sporothallus, which is used to start the modern cultivation of *Porphyra* (Mumford and Miura 1988; Miura 1992). Therefore, from breeding practice and gene bank perspectives, it is very important to maintain sporothalli of various strains of *Porphyra* species.

The gametothalli of *Porphyra* are freezing-resistant (Terumoto 1965; Migita 1966), and the nets with the attached gametothalli in length of 2 to 3 cm could be frozen and preserved at temperatures of -20 - -30°C have been available in the *Porphyra* cultivation industry (Miura 1992). In contrast the sporothalli are very susceptible to freezing in general. Most of sporothallus cells of *P. tenera* were dead when they had placed down to -30°C without cryoprotectants (Migita 1967). A preservation of the sporothalli of *P. yezoensis* at a temperature of -85°C had been tested by Tsuchiya (1989, 1992). The best results of cryopreservation were obtained with the mixture of freezing medium of 10.7% DMSO, 8.6% glucose and 10.7% polyethylene glycol in seawater. In order to get the higher survival rate, it was critical to add the cryoprotectants slowly. The rate of survival was higher at the freezing speed of 0.6°C / minute than 2.4°C / minute. The survival rates of cells in a freezing preservation were gradually decreased and only 13% of the total cells survived after 490 days.

Fujiyoshi *et al.* (1993a, 1993b) have studied on the preservation method at the temperature of liquid nitrogen (LN) by using a two-step cooling method with the sporothalli of *P. yezoensis* f. *narawanensis*. The procedure started with preliminary freezing and followed by the rapid freezing with LN. Adding 1.5 M DMSO and 0.5-0.75 M sorbitol in the seawater of 33‰ to the freezing liquid showed best results. Slow addition of cryoprotectant at 0°C, then preliminary freezing down to -40°C - -85°C at a speed of 0.5-1.0°C / min were critical.

Kuwano *et al.* (1992, 1993) have studied on cryopreservation of sporothalli of *Porphyra yezoensis*; they also studied on cryopreservation of sporothalli of *P. yezoensis, P. tenera, P. pseudolinearis, P. dentata* and *P. haitanensis* by a two-step cooling method. The number of cells survived was maximal when they were prefrozen to -40°C prior to immersion in LN in a cryoprotective solution composed of 10% DMSO and 0.5 M sorbitol in 50% seawater. The survival rates were 38.4-72.5%.

As demand for the preservation of algae has been increasing in the seaweed research and industry, the cryopreservation technique should be one of the best candidates for the various purposes. We initiated to develop a cryopreservation technology for algae in Korea.

MAERIALS AND METHODS

Materials

Sporothalli of *Porphyra seriata, P. yezoensis, P. tenera, P. pseudolinearis* and *P. dentata* from Korea were used in this study. They were maintained as genetic resources at the Aquaculture Research Division of Jeollanamdo Fisheries Research Institute. These free-living sporothalli filaments were cultured in Provasoli's enriched seawater (PES) medium (Provasoli 1966) at 20°C and 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under cool-white fluorescent lamps with a photoperiod of 14:10 h LD. The free-living sporothallus filaments were chopped into short fragments with a Waring blender before use.

Cryoprotectants

Dimethylsulfoxide (DMSO), glycerol, ethylene glycol, proline, hydrochloride betaine, skimmed milk, sucrose, glucose, sorbitol and mannitol were used as cryoprotectants. They were dissolved in seawater (32.0‰) diluted with distilled water to various concentrations. All the solutions were buffered with 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 8.0.

Freezing and thawing

The base solutions were prepared by adding of 0 to 0.1 M (pH was adjusted to 8.0 by adding 0.01 M HEPES buffer) sucrose, glucose, sorbitol or mannitol into a seawater of 32.0‰ with the range of 0-100%. A mixture of about 1 mg sporothalli and 0.75 m*l* base solution were placed in a 2 m*l*-vial and it was cooled in an ice bath. A 0.75 m*l* of sporothallus fragment suspension was added

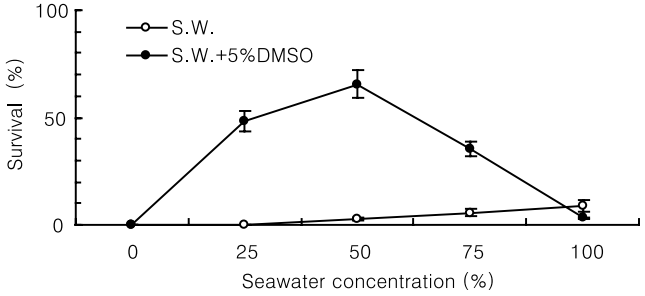

Fig. 1. Effect of the concentration of seawater on survival rate of the sporothalli of *Porphyra seriata*. Sporothallus cells were frozen at -40°C for one day in various concentrations of seawater. Values are means ± SD (n = 3).

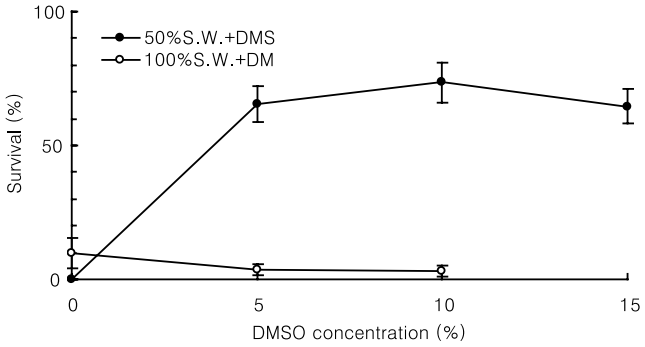

Fig. 2. Effect of the concentration of DMSO on survival rate of the sporothalli of *Porphyra seriata*. Sporothallus cells were frozen at -40°C for one day. As the addition of 30% DMSO to 100% seawater brought about precipitation, the effect of 15% DMSO in 100% seawater was not examined. Values are means ± SD (n = 3).

slowly during a time period of 15 minutes and was stabilized for 45 minutes afterwards.

The cryogenic vial including 1.5 m*l* of the suspension for cryopreservation was frozen at -40°C and -80°C in a programmed freezer (Samwon Freezing Engineering Co.). The temperatures were recorded in the recorder (KA 100, KDNICS). The rate of cooling was slower than 5°C/min. Once algal samples were frozen and preserved in the programmed freezer, it could be thawed by shaking in a water bath at 40°C. When ice in the suspension was mostly melted, the vial was transferred to an ice bath for complete melting of the residual ice.

The suspension was gradually diluted with 10 m*l* of the ice-chilled base solution over a period of 30 min in an ice bath. The diluted suspension was centrifuged at 1,000 rpm for 5 min. The supernatant was removed and the sporothallus fragments were resuspended in 14 m*l* seawater.

The two-step cooling method using a programmed freezer

The solution for the two-step cooling and thawing was made up with 50% seawater (16.0‰) added in 0.5 M sorbitol (pH was adjusted to 8.0 by adding 0.01 M HEPES buffer). A mixture of about 1 mg sporothalli and the 0.75 m*l* solution for the two-step cooling and thawing were placed in a 2 m*l* vial. The vial was placed in the programmed freezer. A 0.75 m*l* of suspension of sporothallus fragments was slowly added during a time period of 15 minutes and was stabilized for 45 minutes afterwards. The vials containing the suspension for cryopreservation were undergone the preliminary freezing down to -40°C at a speed of 1°C/min using a programmed freezer and then immersed into LN. After certain period of the cryopreservation in LN, it could be thawed by shaking strongly in a water bath at 40°C, and put into an ice bath just before ice in the vial was melt down for complete melting of the residual ice. The change of temperatures in the suspension during thawing was monitored with a heat conduction device connected to a recorder. After thawing, the suspension was transferred into a tube of 14 m*l*, and it was diluted by seawater for a time period of longer than 30 minutes by 7 times, and the supernatant was removed after centrifugal separation at 1,000 rpm for 5 minutes. The cryopropectant was removed again by adding the seawater, and the sporothallus fragments were resuspended in seawater.

Measurement of survival ratio

Vitality of the cell was estimated with neutral red staining (Saga *et al.* 1989). The cells were stained with adding the same amount of seawater solution (0.1%) of neutral red into the suspension of sporothallus fragments for 20 minutes, and then the dye was removed by washing with seawater. More than 300 sporothalli were examined under a light microscope. The survival ratio was defined by a relative value of the number of live cells to the dead ones in the sample of the sporothalli after cryopreservation. The mean values were taken from 3 samples processed equally in all the cases.

RESULTS

Cryoprotectants

Fig. 1 shows the survival rate of sporothalli stored at -40°C for one day in various concentrations of seawater with or without DMSO. Without DMSO, the survival

Table 1. The survival rate (mean ± SD) of the sporothalli of *Porphyra seriata* in various cryoprotectants. Sporothalli were frozen at -40°C for one day in each cryoprotectant dissolved in 50% seawater

Cryoprotectant	Survival rate (%)
Control (50% seawater)	2.1 ± 2.1
5% DMSO	65.4 ± 4.8
5% Ethylene glycol	3.2 ± 3.1
5% Glycerol	6.8 ± 4.2
5% Proline	18.3 ± 4.6
5% Betaine·HCl	9.7 ± 2.4
5% Skimmed milk	1.8 ± 1.3
1 M Glucose	4.6 ± 3.2
1 M Sucrose	43.1 ± 4.9
1 M Sorbitol	3.5 ± 2.6
1 M Mannitol	1.6 ± 1.4

rates were lower than 10% and decreased with the dilution of seawater. With DMSO (5%), the survival rates varied with the concentrations of seawater, and it was the highest of 65.4% at 50% seawater. As shown on Fig. 2, the increase in DMSO concentration (10%) in 100% seawater gave no increase in the survival rate. In 50% seawater DMSO had cryoprotective effect at concentrations up to 15%, but the increase in DMSO concentration from 5 to 15% did not give any increase in the survival rate. Based on the results in Fig. 1 and 2, 50% seawater was applied in the following experiments.

Table 1 shows the survival rate of sporothalli in each cryoprotectant. DMSO, sucrose and proline had shown good cryoprotective effect, whereas others had little or none. DMSO was the best cryoprotectant among the compounds examined. Sucrose showed cryoprotective effect between 0.1 and 1 M (Fig. 3). When combined with DMSO, however, the survival rate decreased with increasing sucrose concentration. Sorbitol had no cryoprotective effect between 0.1 and 1.0 M, and the combination of sorbitol with DMSO did not show any increase in survival rate as compared with 5% DMSO alone (Tables 1 and 2, and Fig. 3).

Change in the survival rate of the sporothallus cells stored at -40°C are shown in Table 2. When the sporothallus cells were frozen in 5% DMSO or in the mixture of 5% DMSO and 0.1 M sucrose, about 65% of the cells survived for one day, and the survival rates decreased below 1% in a week. When frozen in 5% DMSO combined with 0.5 M sorbitol, 53% of the sporothallus cells survived for one day, and 14% of the cells survived for a week. The survival rate fell below 1%

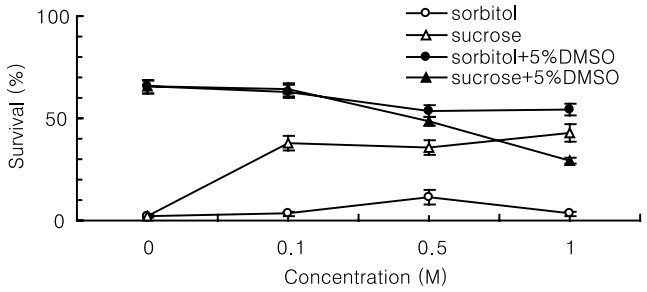

Fig. 3. Effect of the sorbitol and sucrose concentrations on survival rate of the sporothalli of *Porphyra seriata* frozen at -40°C for one day. Cryoprotectants were added in 50% seawater. Values are means ± SD (n = 3).

in 60 days.

Table 3 shows the survival rate of the sporothallus cells stored at -80°C for various periods of time. When the sporothallus cells were frozen in 5% DMSO or in the mixture of 5% DMSO and 0.1 M sucrose, the survival rate fell below 1% in a day. When frozen in 5% DMSO combined with 0.5 M sorbitol, the survival rate fell below 1% in three days.

Two-step cooling method

When sporothallus cells were frozen from -20 to -80°C at a rate of 1°C/min and immediately thawed, the survival of sporothallus cells decreased with decreasing temperature (Fig. 4A). Increasing DMSO concentration reduced the decrease in survival. With 15% DMSO, however, the survival of sporothallus cells was lower especially in the case of those cooled to -20 - -40°C possibly due to an inhibitory effect of DMSO at high concentration. The survival of sporothallus cells immersed in LN after prefreezing to -20 and -30°C was less than 20% (Fig. 4B). The survival rate was highest, however, when sporothallus cells were prefrozen to -40°C and immersed in LN (Fig. 4B). Prefreezing to -50 - -80°C followed by immersion in LN decreased the survival gradually with the decrease in prefreezing temperatures. Regardless of DMSO concentrations, the optimal prefreezing temperature was -40°C.

Fig. 5 shows the survival of sporothallus cells which were cooled to -40°C at 1°C/min with 0-20% DMSO and thawed before or after the immersion in LN. The optimal DMSO concentration was deduced to be 10%, thus DMSO was added to a final concentration of 10% in the following experiments.

The difference in cooling rate (0.5-4°C/min) had little effect on the survival of sporothallus cells thawed immediately from -20 to -80°C (Fig. 6A). When immersed

Table 2. The survival rate (mean ± SD) of the sporothalli of *Porphyra seriat*a stored at -40°C for different periods of time. Cryoprotectants were added in 50% sea water

Cryoprotectant	Survival rates (%)						
Cryoprotectant	1 hours	1 day	7 days	10 days	20 days	40 days	60 days
5% DMSO	-*	65.4 ± 4.8	0.5 ± 0.5	0.1 ± 0.1	-	-	-
5% DMSO + 0.5 M sorbitol	75.2 ± 1.8	53.6 ± 3.6	14.2 ± 4.2	9.3 ± 3.8	3.2 ± 0.4	1.5 ± 0.7	0.4 ± 0.4
5% DMSO + 0.1 M sucrose	-	48.6 ± 6.9	0.4 ± 0.4	0.1 ± 0.1	-	-	-

* -: not examined.

Table 3. The survival rate (mean ± SD) of the sporothalli of *Porphyra seriata* frozen at -80°C for different periods of time. Cryoprotectants were added in 50% seawater

Cryoprotectant	Survival rates (%)			
	4 hours	1 day	3 days	7 days
5% DMSO	-*	0.2±0.2	-	0.2±0.2
5% DMSO+0.5M sorbitol	1.8±0.7	1.5±0.4	0.4±0.3	0
5% DMSO+0.1M sucrose	-	0	-	0

* -: not examined.

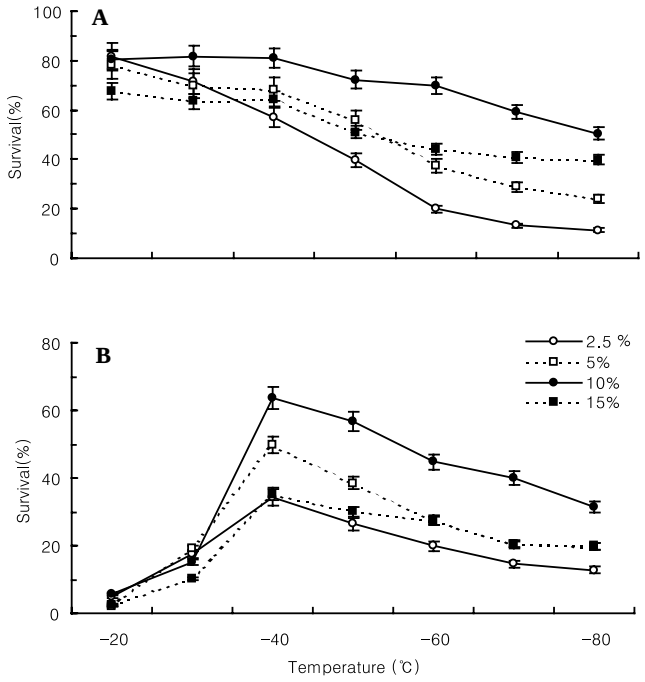

Fig. 4. The survival rate of the sporothalli of *Porphyra seriata* cooled to various temperatures at 1°C/min. Sporothallus cells were suspended in cryoprotective solutions composed of 2.5-15% DMSO and 0.5 M sorbitol in 50% seawater. A, thawed immediately after prefreezing; B, stored for one day in LN before thawing. Values are means ± SD (n = 3).

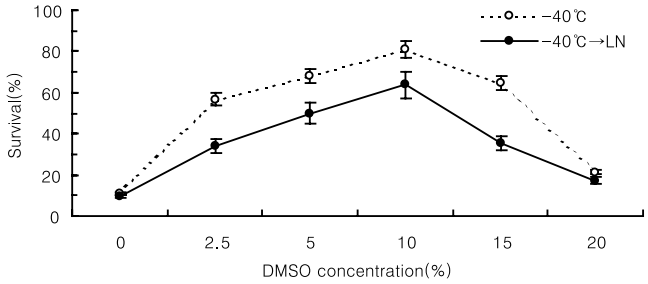

Fig. 5. Effect of DMSO concentrations on survival of the sporothalli of *Porphyra seriat*a. Sporothallus cells were suspended in cryoprotective solutions composed of 0-20% DMSO and 0.5 M sorbitol in 50% seawater, cooled to -40°C at 1°C/min and thawed before or after the immersion in LN for one day. Values are means ± SD.

in LN, however, the optimal prefreezing temperature differed with cooling rates, being -40°C at 0.5 and 1°C/min and -50°C at 2 and 4°C/min (Fig. 6B). The highest survival was obtained when sporothallus cells were prefrozen to -40°C at 0.5°C/min.

Fig. 7 shows the survival of sporothallus cells frozen to -40°C at various cooling rates (0.1-4°C/min) and thawed before or after the immersion in LN. The increase in cooling rates above 1°C/min was considered to reduce sporothalli survival. Retention of sporothallus cells for various periods of time (up to 60 min) at -40°C after cooling to this temperature at rates of 0.5-2°C/min gave no change in the survival regardless of cooling rates (Fig. 8). Based on the results in Fig. 6-8, sporothallus cells were cooled to -40°C at 1°C/min and immediately immersed in LN in the following experiments.

The survival of sporothallus cells thawed at different

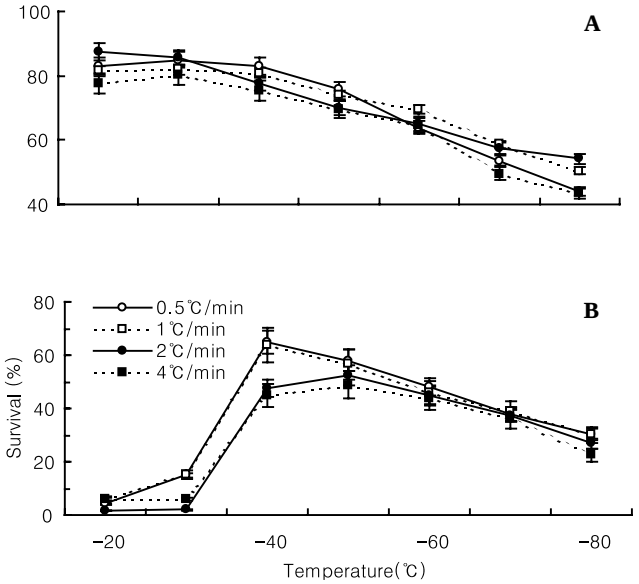

Fig. 6. Effect of cooling rates on survival of the sporothalli of *Porphyra seriata*. Sporothallus cells were frozen to -20 - -80°C at 0.5-4°C / min and thawed before (A) or after (B) the immersion in LN for one day. Values are means ± SD (n = 3).

Table 4. Comparison of the survival (mean ± S.D.) of sporothallus cells of *Porphyra seriata* when sporothalli suspensions with cryoprotectants for the two-step cooling experiment were thawed at different condition. The frozen suspensions were thawed by agitation of vials in a water bath at 40°C, by keeping vials in a water bath at 0°C, or by keeping vials in air at 20 or 4°C

Thawing	Survival (%)	
	-40°C	-40°C → LN
40°C (in water)	80.9 ± 4.6	63.8 ± 3.8
0°C (in water)	78.6 ± 5.4	45.5 ± 4.2
20°C (in air)	77.4 ± 2.8	25.1 ± 4.5
4°C (in air)	75.6 ± 5.9	7.9 ± 3.8

warming rates was summarized in Table 4. The frozen sporothallus suspensions were thawed by agitating vials in a water bath at 40°C, by keeping vials in a water bath at 0°C, or by keeping vials in air at 20°C or 4°C. The warming rate decreased in this order. The difference in warming rates had no effect on the survival of sporothallus cells which were thawed from -40°C, whereas the decrease in warming rates reduced the survival of sporothallus cells stored in LN.

The survival was higher when DMSO was gradually added to reach its final concentration compared to when sporothallus cells were immediately suspended in the cryoprotective solution having 10% DMSO. However, no

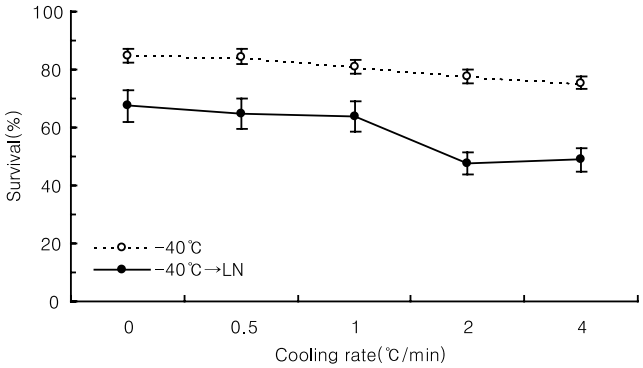

Fig. 7. Effect of cooling rates on survival of the sporothallus of *Porphyra seriata*. Sporothallus cells were cooled to -40°C at 0.1-4°C / min and thawed before or after the immersion in LN for one day. Values are means ± SD (n = 3).

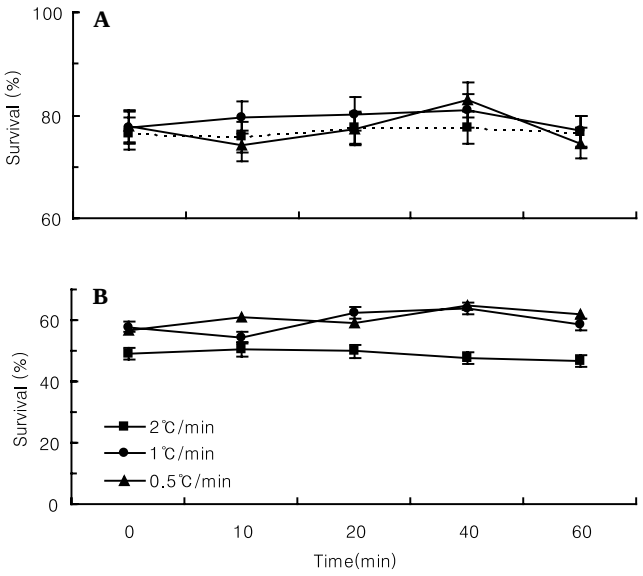

Fig. 8. Effect of retention for various periods of time at -40°C on survival of the sporothalli of *Porphyra seriata*. Sporothallus cells were cooled to -40°C at 0.5-2°C / min and retained for various periods of time. A, thawed immediately after prefreezing; B, stored for one day in LN before thawing. Values are means ± SD (n = 3).

favorable effect was found on the survival if DMSO was added slowly over a period of 30 min as compared with the case of 15 min (Table 5).

Fig. 9 shows the survival of sporothallus cells, which were equilibrated for various periods of time (5-240 min) after addition of DMSO. The difference in equilibration period had no effect on the survival.

Table 6 shows the survival of the sporothallus cells for different dilution rates of the cryoprotective solution after thawing. When the sporothallus suspension with cryoprotectants was diluted in 7 fold with a single

Table 5. Comparison of the survival (mean ± SD, n = 3) of sporothallus cells of *Porphyra seriata* when DMSO was added at different time periods. An equal volume of the solution for the two-step cooling experiment having 20% DMSO was gradually added over a period of 15 or 30 min

DMSO addition[a] (min)	Survival (%)	
	-40°C	-40°C → LN
0[b]	48.3 ± 4.6	40.1 ± 4.3
15	79.2 ± 4.1	62.9 ± 5.9
30	80.9 ± 2.8	63.8 ± 3.8

[a] Time required to attain a final concentration of 10%.
[b] Sporothallus cells were centrifuged and suspended immediately in the cryoprotective solution having 10% DMSO.

Table 6. Comparison of the survival (mean ± SD, n = 3) of sporothallus cells of *Porphyra seriata* for different dilution rates of the cryoprotective solution. After thawing, sporothalli suspensions with cryoprotectants for the two-step cooling experiment were diluted 7-fold with ice-chilled seawater over various periods of time

Dilution time (hr)[a]	Survival (%)
Rapid dilution[b]	55.5 ± 5.4
0.5	63.8 ± 3.8
1.5	58.1 ± 6.2
3.5	60.2 ± 7.8

[a] Time required to dilute 7-fold with seawater.
[b] Sporothalli suspension with cryoprotectants was diluted at 7-fold with a single addition of seawater.

addition of ice-chilled seawater, the survival reduced slightly. No effect was observed on the survival if the period of dilution was prolonged from 0.5 hr to ~ 3.5 hr.

Cryopreservation of sporothalli of 4 species of *Porphyra*

Since the optimal conditions of the cryopreservation according to the two-step cooling method became clear through various investigations using *Porphyra seriata* as mentioned before about the cryopreservation of the sporothalli in the *Porphyra*, the survival rate of the sporothalli in the four species of *Porphyra* (*P. tenera, P. yezoensis, P. pseudolinearis* and *P. dentata*) in addition to *P. seriata* were evaluated after the cryopreservation. Sporothalli were minutely cut with a homogenizer, mixed with freezing media containing 5% or 10% of DMSO and 0.5% of sorbitol in 50% sea water, frozen to -40°C at 1°C/min using the programmed freezer in

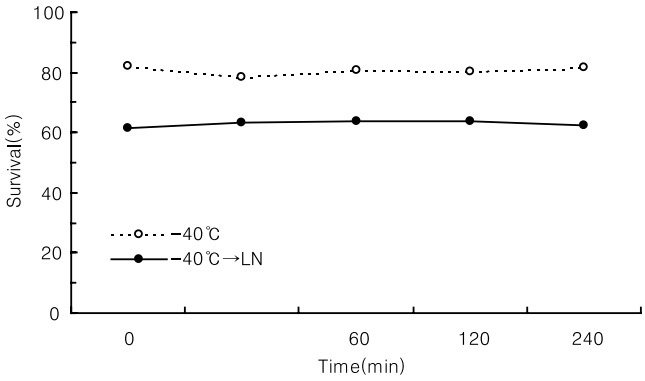

Fig. 9. Effect of equilibration for various periods of time after DMSO addition on survival of the sporothalli of *Porphyra seriata*. Values are means ± SD (n = 3).

Table. 7. Comparison of the survival (mean ± SD, n = 3) of sporothallus cells of *Porphyra*s prefrozen to -40°C in a cryoprotective solution of 10% DMSO and 0.5M sorbitol in 50% seawater by a programmable freezer prior to immersion in LN

Species	Survival (%)
Porphyra seriata	63.8 ± 3.8
P. yezoensis	61.0 ± 2.5
P. tenera	54.6 ± 5.3
P. pseudolinearis	58.4 ± 7.2
P. dentata	70.9 ± 3.3

advance and cooled suddenly down to the LN temperature for one day to compare the survival rate (Table 7). When using the programmed freezer (at 1 °C/min), *P. dentata* and *P. yezoensis* showed higher survival rate than *P. tenera* and *P. pseudolinearis*.

DISCUSSION

Gametothalli of *Porphyra* were highly resistant to frost, and showed high rates of survival when cooled below -40°C even without cryoprotectants (Migita 1964, 1966; Kuwano *et al.* 1996). On the other hand, the sporothalli of *P. tenera* was sensitive to frost, and perished when cooled to -30°C (Migita 1967). As shown in the present study, the sporothalli of *P. seriata* was also sensitive to frost without cryoprotectants. DMSO, which is often used as a cryoprotectant for higher plant cells (Sakai and Sugawara 1978; Ulrich *et al.* 1979) showed a cryoprotective effect on the sporothalli of *P. seriata*. However, the cryoprotective effect of DMSO varied with the concentrations of seawater. Some marine algae were successfully cryopreserved in the media with half

strength of ambient seawater salinity when added with cryoprotectants (van der Meer and Simpson 1984). In *P. seriata*, the cryoprotective effect of DMSO was also the highest at a seawater concentration of 50%.

DMSO was most favorable for the survival of sporothalli of *P. seriata* at -40°C among the cryoprotectants tested in this study. Although glycerol has been known as a good cryoprotectant for bacteria and microalgae (Bousfield 1984; Leeson *et al.* 1984), but it showed only a little cryoprotective effect on the sporothalli of *P. seriata* in this study.

For successful cryopreservation in higher plants, sugars or sugar alcohols have often been mixed with DMSO (Ulrich *et al.* 1979; Chen *et al.* 1984a). In *P. seriata*, however, the combination of sucrose with DMSO was not favorable for cryopreservation of *Porphyra* sporothalli, even though sucrose, when used singly, was effective to some extent. The combination of sorbitol with DMSO was effective for the sporothalli frozen at -40°C and they survived longer as compared with DMSO alone.

The mixed solution composed of 5% DMSO and 0.5M sorbitol in 50% seawater showed the most favorable cryoprotective result with the sporothalli of *P. seriata*. However, the storage of the sporothalli at -20 or -80°C in a freezer was successful only for a short period of time, as the survival rates in this solution at -20 or -80°C fell below 1% in three days or in two months.

Cells of higher plants and algae were successfully cryopreserved by the two-step cooling method combining slow cooling to -40°C and rapid cooling to -196°C by immersion in LN (Bajaj and Reinert 1977; Chen *et al.* 1984a; Lesson *et al.* 1984; van der Meer and Simpson 1984). Morris (1978) reported that the cells of *Chlorella emersonii* which had survived from the slow cooling to -40°C and also survived mostly after storage in LN. Kuwano *et al.* (1992) reported the cryoprotective effects of cryoprotectants on the sporothalli of *P. yezoensis* and showed that the sporothalli of *P. yezoensis* cooled to -40°C survived at high rates in the adequate solution. The two-step cooling method was also successful in cryopreservation of the sporothalli of *P. seriata* in this study.

Generally an application of the cryoprotective solution is critical in cryopreservation of plant cells (Bajaj and Reinert 1977). The cryoprotective solution composed of 5% DMSO and 0.5 M sorbitol in 50% seawater had been proved to show favorable effect on cryopreservation of the sporothalli of *P. yezoensis* (Kuwano *et al.* 1992, 1993, 1994). The increase of DMSO concentration to 10% gave favorable effect on the survival of the sporothallus cells of *P. seriata.* A solution composed of 10% DMSO and 0.5 M sorbitol in 50% seawater was most effective mixture for cryopreservation of the sporothalli of *P. seriata.* As in the cases of Sugawara and Sakai (1974) and Chen *et al.* (1984a, 1984b) DMSO should be added gradually to the final concentration over a period of 15 min, otherwise the survival of sporothallus cells was reduced. It is generally considered that DMSO permeates into cells and prevents the cells from freezing injury (Bajaj and Reinert 1977). Therefore, cells need a sufficient period of time after addition of DMSO for equilibration (Chen *et al.* 1984b). DMSO could permeate easily into the sporothallus cells of *P. seriata*, 5 min was found sufficient for the equilibration in this study.

Successful cryopreservation of higher plants and algae has been achieved by prefreezing at 0.3-6°C / min from -30 to -40°C prior to immersion in LN (Chen *et al.* 1984b; van der Meer and Simpson 1984). When cell suspensions are cooled slowly, ice forms in the external medium but the cell contents remain unfrozen and supercooled, and water flows out of the cells and freezes externally because of difference in chemical potential between water in the cells and that in the partly frozen solution outside the cells (Mazur 1984). It is generally considered that such appropriately dehydrated cells would be able to survive at the LN temperature. Rapid cooling may prevent the dehydration (Chen *et al.* 1984b). In *P. seriata*, appropriate cooling rates were 0.1-1°C / min and optimal prefreezing temperature was at -40°C. Sporothallus cells prefrozen to temperature above -40°C were damaged mostly during the rapid cooling in LN. It is possible that sporothallus cells cooled to temperatures above -40°C may not have been dehydrated enough to survive at the LN temperature.

Although prefreezing to -40°C or below may have reduced cell damage during rapid cooling, cell damage during prefreezing became progressively greater with decrease in prefreezing temperature. Reduction of the survival during prefreezing seems to be due to the decrease in temperature rather than to the extension of exposure time to sub-zero temperatures since both the decrease in cooling rates and retention at -40°C did not reduce the survival. Morris (1978) reported that retention at -30°C for a suitable period of time following slow cooling to this temperature gave increase in the cell survival of microalgae. On the other hand, retention at -40°C gave no increase in the survival in this study. To

avoid extreme supercooling, ice nucleation was usually induced artificially (Sugawara and Sakai 1974). It is generally considered that the cells stored in LN should be warmed rapidly, otherwise the ice crystals formed in the cells during rapid cooling grow to a harmful size, resulting in cell death during thawing (Sugawara and Sakai 1974). In *P. seriata*, the decrease in warming rates reduced the survival of sporothallus cells stored in LN, but had no effect on that of sporothallus cells thawed immediately from -40°C. The cell contents could be remain unfrozen at -40°C, and then be frozen by immersion in LN.

Post-thaw removal of cryoprotectants was another critical step in cryopreservation of higher plants (Chen *et al.* 1984b). In *P. seriata*, to minimize the damage by dilution, sporothallus suspension containing cryoprotectants was gradually diluted by 7-fold with ice-chilled seawater over a period of 30 min. However, reduction in the survival by rapid dilution was not significant. It has been generally considered that cell survival was independent of the period of storage at the LN temperature (Morris 1978). Kuwano *et al.* (1993) reported the survival of sporothallus cells was not reduced for at least 300 days. It is likely that sporothallus cells can be stored in LN for a much longer period.

Van der Meer and Simpson (1984) succeeded in cryopreservation of several species of marine macroalgae in LN, and determined which of the known factors were critical in cryopreservation in LN. They assessed the viability of plants by regeneration of frond tips or 'pigmentation index'. However, assessment by the regeneration of frond tips was not sufficiently sensitive, and that by 'pigmentation index' was subjective. The development of cryopreservation of marine macroalgae requires careful studies of sensitive and objective assays.

Optimal conditions for cryopreservation of the sporothalli of *P. yezoensis* was cooling rates of 0.1-1°C $\cdot$ min^{-1} and prefreezing temperature at -40°C. The retention time (up to 60 min) at -40°C after cooling to this temperature resulted in no change in survival, and the temperature for ice nucleation did not influence survival (Kuwano *et al.* 1993). Cryopreservation by the two-step cooling method requires equipment that regulates prefreezing rates. A programmable freezer could accurately regulate the cooling rate and automatically induce ice nucleation.

The technique described here will be applicable to other species / strains of *Porphyra*; however, attention should be given to the prefreezing temperature and composition of the cryoprotective solution, especially the DMSO concentration. Prefreezing temperature is critical in cryopreservation by the two-step cooling method. Sporothallus cells prefrozen to temperatures above the adequate one will be damaged considerably when immersed in LN. Successful cryopreservation of various cultured cells was accomplished by prefreezing to -30 to -40°C (Morris 1978; Sugawara and Sakai 1974; Ben-Amotz 1980a, 1980b; Chen *et al.* 1984b; van der Meer and Simpson 1984; Day and Fenwick 1992). Fahy *et al.* (1984) predicated an optimum prefreezing temperature of -40°C because it may be sufficiently low enough at this temperature to lower the homogeneous nucleation temperature of cytoplasm to near the glass transition temperature of the cytoplasm. If the adequate prefreezing temperature were below -40°C for the cryopreservation of other species of *Porphyra*, the survival after immersion in LN would be considerably lower than that after thawing from -40°C, mainly because of insufficient dehydration during prefreezing. DMSO concentration also affects the survival of sporothallus cells. Cryopreservation of various cultured cells was accomplished successfully in cryoprotective solutions having 5-10% DMSO (Bajaj and Reinert 1977; Lesson *et al.* 1984). The optimal concentration of DMSO was 10% for all species of *Porphyra* used in this study. However, it should be reexamined for other species / strains of *Porphyra*. Kuwano *et al.* (1994) reported that the survival rates of strains of *P. yezoensis* and three species of *Porphyra* from Japan and one species of *Porphyra* from China were different under the same condition.

The survival rates of the present study were 54.6-70.9%, and those of Kuwano *et al.* (1994) were 38.4-72.5% when a programmable freezer was used. These results were almost the same except the lowest survival rate when a yellow-type pigmentation mutant of *P. yezoensis* was used as a material. They also reported a cryopreservation method by applying a simple prefreezing system and the survival rates were 44.3-77.9%. They were compared with the survival rates of 38.4-72.5% by using a programmable freezer. This method should be reexamined for other species / strains of *Porphyra* from Korea.

In conclusion, the present study by the cell survival assay proposes an appropriate procedure for the cryopreservation of the sporothalli of *Porphyras* as followings. Sporothallus fragments are suspended in a solution that is composed of 0.5 M sorbitol in 50% seawater. To this suspension, an equal volume of the

solution having 20% DMSO is added gradually over a period of 15 min followed by an equilibration period of several minutes. The sporothalli suspension with cryoprotectants is preforzen to -40°C at 0.1-1°C / min, and immersed in LN. After storage in LN, the suspension is thawed quickly in a water bath at 40°C, and the vial containing the suspension put into an ice bath for complete melting of the residual ice. The cryoprotectant is washed off by gradual dilution with seawater.

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