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Cryopreserved Marine Microalgae Grown Using Different Freezing Methods

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Marine microalgae are a key diet component in finfish and shellfish aquaculture. Cryopreservation of the microalgae is suggested by many other studies as the best method for long-term storage. To test cryopreservation efficacy, 19 taxas of marine microalgal species were examined. In the first experiment we compared dimethylsulfoxide (Me_2SO) and glycerol, which are most widely used as cryoprotectant agents (CPAs). The cryopreservation comprised two freezing procedures. Firstly, the samples containing the CPAs were kept at 4°C for 10 min before being plunged into liquid nitrogen (-196°C). Secondly, samples containing CPAs were pre-cooled (-1°C min^{-1}) to -80°C before being plunged into liquid nitrogen. Most of the species were successfully cryopreserved using Me_2SO, whereas the Prasinophyceae (*T. striata* and *T. suecica*) were successfully cryopreserved using glycerol. In general, the cooling method had no influence on the survival of the microalgae except in the case of the *Tetraselmis* species. In the second experiment, the cultured solution was divided before cryopreservation into concentrated and non-concentrated groups to identify the effect of cell density during cryopreservation. After 12 months of storage, the samples were again divided into centrifugation and non-centrifugation groups to learn the effect of Me_2SO on the culture. Viability and growth of the microalgae were not influenced by cell density or the centrifugal removal of the Me_2SO after thawing.

Key Words: cryopreservation, dimethylsulfoxide (Me_2SO), glycerol, marine microalgae

INTRODUCTION

Marine microalgal species are widely used as a food source in the artificial propagation of hatchery-reared mollusks, crustaceans and fish (Cañavate and Lubian 1995b) and could be the source of diverse new products and medicines (Rhodes *et al.* 2006). For this reason, special control of microalgal species is needed to ensure long-term storage free of any contaminants.

One preservation method for microalgae is to use a liquid or agar medium. Compared to freezing (0°C to -20°C), which is effective for only about two months (Terauchi *et al.* 1997), storage is possible for up to a year when an agar medium is used. But long-term storage of subcultures runs the risk of contamination from different organisms and genetic mutation (Nagasaki 2001). Therefore, a long-term preserving method for microalgae is important. Recently in aquaculture, drying, freeze-drying, and cryopreservation techniques have been explored. However, drying and freeze-drying have not been very successful for the long-term storage of algae (Taylor and Fletcher 1998).

Cryopreservation has been successfully used in animals and plants (Taylor and Fletcher 1998). Reports on the cryopreservation of microalgae have recently increased (Poncet and Véron 2003; Houdan *et al.* 2005). Cryopreservation entails preparing the cultures and then storing them in liquid or vapor-phase nitrogen. These methods require significant initial effort, but in the long-term reduce labor requirements and opportunities for contamination. In addition, because these are long-term storage methods, genetic diversity is maintained.

A few freshwater microalgal species and some marine diatoms have been successfully cryopreserved, but it is widely recognized that many microalgal species are recalcitrant to standard cryopreservation techniques (Cañavate and Lubian 1995a, 1995b, 1997a, 1997b; Tzovenis *et al.* 2004). The importance for the survival of the microalgae of the interaction between the rate of cooling, the cryoprotective additive and the rate of warming has been stressed (Mazur 1969).

This study describes differences in the viability and growth of marine microalgae when subjected to different

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cryoprotectant agents (CPAs), cooling methods, cell densities at the time of cryopreservation, and the removal of the dimethylsulfoxide (Me_2SO) after thawing.

MATERIALS AND METHODS

Organisms and culture conditions

In the first experiment on cryopreservation freezing procedures, 12 marine microalgal species were examined (Table 1): *Gloeocystis gigas*, *Nannochloris oculata*, *Stichococcus bacillaris*, *Nannochloropsis oceanica*, *N. salina*, *N.* sp., *Chrococcus minutus*, *Oscillatoria angustissima*, *Synechococcus nidulans*, *Trichodismium erythraeum*, *Tetraselmis striata* and *T. suecica*. All species were maintained at the Korea Marine Microalgae Culture Center (KMMCC). The microalgae were grown in 250 mL Erlenmeyer flasks containing 50 mL of f/2 medium filtered seawater (33 psu, Guillard and Ryther 1962) at 20°C with continuous illumination of 60 μmol photon m^{-2} s^{-1} provided by white fluorescent light without aeration for 7 days.

To test the effect of cell density during cryopreservation and the removal of Me_2SO after thawing on the viability and growth of the microalgae, 11 microalgal species at the KMMCC were examined (Table 2): *Chaetoceros gracilis*, *Navicula annexa*, *Phaeodactylum tricornutum*, *Skeletonema costatum*, *N. oculata*, *Spirulina maxima*, *N. oceanica*, *T. striata*, *T. suecica*, *Isochrysis galbana*, and *Pavlova pinguis*. The culture condition of the microalgae was the same as those mentioned-above except for *S. maxima*, which was cultivated in Spirulina medium (Schlösser 1982). To test the effect of cell density during cyropreservation, two samples were used as a control group: one in which the cell density of the culture was concentrated three times, the other in which the culture was non-concentrated. For this test, liquid cultures were harvested at exponential growth phase by centrifucation (1,000 rpm, 5 min for *Spirulina* and 1,500 rpm, 10 min for others species). The concentrated microalgae were washed with the fresh medium three times and the pellets were resuspended in fresh medium.

Cryopreservation procedure

In the first experiment, we used two different cryoprotectant agents: dimethylsulfoxide (Me_2SO, 10%) and glycerol (10%). Filtered Me_2SO was added to the autoclaved f/2 medium. Glycerol was added to the f/2 medium, which was then autoclaved. Samples of the culture (0.5 mL) were dispensed into cryovials (T310-2A 2 mL, Simport, Canada) and incubated at 4°C for 10 min prior to cryopreservation. Cryopreservation consisted of two freezing methods: one, the samples containing the CPAs were transferred and held at 4°C for 10 min before being plunged into liquid nitrogen (-196°C); two, the samples containing CPAs were transferred to hold at 4, -20 and -80°C for 10 min, respectively before being plunged into liquid nitrogen. Their viability and growth were tested after 1 and 6 months of storage.

Table 1. Micoralgal species used in the experiment on the effect of cryoprotective agents and freezing procedures

	Species	Source of strain
Chlorophyceae	*Gloeocystis gigas*	KMMCC-C 91
	Nannochloris oculata	KMMCC-C 31
	Stichococcus bacillaris	KMMCC-C 2
Cyanophyceae	*Oscillatoria angustissima*	KMMCC-CY 3
	Chrococcus minutus	KMMCC-CY 40
	Synechococcus nidulans	KMMCC-CY 32
	Trichodesmium erythraeum	KMMCC-CY 16
Eustigmatophyceae	*Nannochloropsis oceanica*	KMMCC-EUS 7
	Nannochloropsis salina	KMMCC-EUS 1
	Nannochloropsis sp.	KMMCC-EUS 6
Prasinophyceae	*Tetraselmis striata*	KMMCC-P 5
	Tetraselmis suecica	KMMCC-P 4

KMMCC, Korea Marine Microalgae Culture Center.

In the second experiment, 10% Me_2SO was used for the CPA. The samples containing the Me_2SO was kept at 4°C for 10 min before being plunged into liquid nitrogen (-196°C). Their growth was tested after 12 months of storage in liquid nitrogen.

Thawing procedure

After one and six months of storage, samples of the frozen paste were extracted from the liquid nitrogen and transferred immediately to a water bath at 35°C for 5 min. To test the viability, cells were thawed at 35°C until the ice was completely melted. Thawed samples were diluted ten-fold in fresh f/2 medium. The samples were incubated at 20°C with an illumination of 60 μmol photon m^{-2} s^{-1}. The cells were counted on a Neuvauer haemacytometer for 15 days and then we analyzed the daily specific growth rate (s.g.r = $3.322 \times \log(N_1/N_0)/t_1-t_0$) (Guillard 1973) during log phase growth. Motile Prasinophyceae (*T. striata* and *T. suecica*) were treated with 1% formaldehyde and the total number of cells was counted. All the experiments were performed in triplicate.

After 12 months of storage, the cells were thawed by

Table 2. Micoralgal species used in the experiment on the effect of cell density in cryopreservation and removal of Me_2SO after thawing

	Species	Source of strain
Bacillariophyceae	*Chaetoceros gracilis*	KMMCC-B 15
	Navicula annexa	KMMCC-B 81
	Phaeodactylum tricornutum	KMMCC-B 44
	Skeletonema costatum	KMMCC-B 285
Chlorophyceae	*Nannochloris oculata*	KMMCC-C 31
Cyanophyceae	*Spirulina maxima*	KMMCC-CY 23
Eustigmatophyceae	*Nannochloropsis oceanica*	KMMCC-EUS 7
Prasinophyceae	*Tetraselmis striata*	KMMCC-P 5
	Tetraselmis suecica	KMMCC-P 4
Prymnesiophyceae	*Isochrysis galbana*	KMMCC-H 2
	Pavlova pinguis	KMMCC-H 14

KMMCC, Korea Marine Microalgae Culture Center.

the same method mentioned-above. The samples were divided into two groups to learn the effect of the Me_2SO on the culture. The thawed samples were directly diluted in fresh f/2 or Spirulina medium. The other samples were harvested by centrifugation (5,000 rpm, 3 min) and washed once with fresh medium to remove the Me_2SO. The pellets were resuspended in fresh medium and cultured. The cells were cultured by using the previous method. The cells were counted every 5 days for 60 days and daily s.g.r was analyzed during log phase growth. Motile Prasinophyceae (*T. striata*, *T. suecica*) and Prymnesiophyceae (*I. galbana*, *P. pinguis*) were treated with 1% formaldehyde and the total number of cells was counted. All the experiments were performed in triplicate.

Statistical analysis

The statistical significance level was analyzed by one-way analysis of variance (ANOVA) (Duncan's multiple range tests; Duncan 1955). Statistical significance was determined at $p < 0.05$. Statistical analysis of data included the Student's *t*-test. All statistics were conducted by SPSS software (version 10.1; SPSS Inc., Chicago, IL, USA).

RESULTS

Effect of CPAs and cooling methods Growth after cryopreservation for one month

Most of the species were successfully cryopreserved using 10% Me_2SO (Fig. 1). Growth of *N. oceanica* proved best following a rapid cooling from 4°C and then a plunge into liquid nitrogen with the addition of Me_2SO. Its cell density reached to 3433.3 x 10^4 cells mL^{-1}. However, when using glycerol, growth differed depending on the freezing procedure and microalgae growth was very low with 129.1 x 10^4 cells mL^{-1} when the rapid cooling method was used. The effects of freezing procedures on the growth of cryopreserved *N. salina* were not different in Me_2SO. The effects of slow and rapid cooling on the growth of cryopreserved *N.* sp. were different in Me_2SO. But no growth was found in glycerol. *N. oculata* grew only following rapid cooling in Me_2SO. *G. gigas* grew best in Me_2SO with slow cooling. The cooling procedure did not affect the growth of *G. gigas* in glycerol. The effects of slow and rapid cooling on the growth of cryopreserved *S. bacillaris* were not different in any of the CPAs. But Me_2SO showed higher growth than glycerol did.

C. minutus grew to 228.5 x 10^4 cells mL^{-1} in slow cooling with Me_2SO. *O. angustissima* was successfully cryopreserved in Me_2SO, but the effect of cooling method was not distinct. *S. nidulans* and *T. erythraeum* did not grow in glycerol. The effects of slow and rapid cooling on the growth of these microalgal species were different when tried in Me_2SO. The cell density of *S. nidulans* in slow cooling with Me_2SO reached to 1365.8 x 10^4 cells mL^{-1}. Although the growth was low in *Tetraselmis*, the addition of glycerol in rapid cooling method increased the cell density of *T. suecica* to 39.0 x 10^4 cells mL^{-1}.

The daily specific growth rate of each microalgal group by class in this experiment is shown in Table 3. In general, Me_2SO produced significantly higher growth rate than glycerol ($p < 0.05$); however, the cooling method had no affect on the growth of the microalgae. In Eustigmatophyceae and Cyanophyceae, cryopreservation with Me_2SO showed significantly higher growth rate than that with glycerol ($p < 0.05$). In Chlorophyceae, the choice of the CPA and cooling method had no affect on the growth of the microalgae. Cryopreservation of Prasinophyceae was more difficult than that for the other microalgal groups.

Growth after six-month of cryopreservation

The results on growth after cryopreservation for six month is shown in Fig. 2. Although *N. oceanica* grew well to the density of 3340 x 10^4 cells mL^{-1} in Me_2SO following the rapid cooling method, it grew badly in glycerol and the freezing methods were not distinct. The effects of slow and rapid cooling on the growth of cryopreserved *N. salina* and *N.* sp. were not distinct in Me_2SO. But glyc-

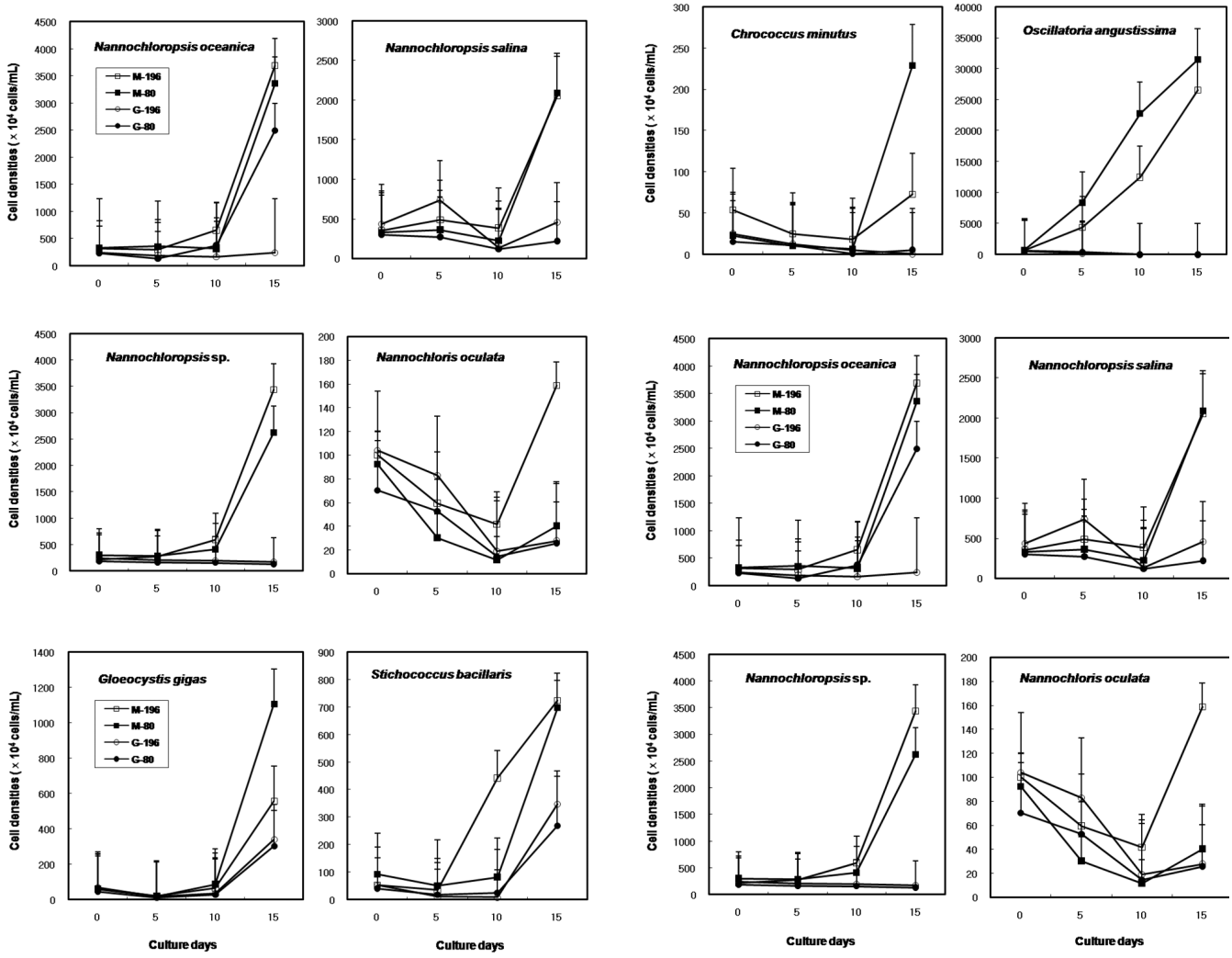

Fig. 1. Growth of each microalgal species cryopreserved with different cryopreservative agents and cooling methods for one month (M, Me_2SO; G, Glycerol. -196, kept in -196°C directly; -80, kept in -196°C after keeping in -20°C and -80°C for 10 min, respectively).

erol showed bad growth. *N. oculata* grew best in Me_2SO following the rapid cooling method. *G. gigas* was successfully cryopreserved using all cryoprotectant agents and freezing procedures. The effects of slow and rapid cooling on the growth of cryopreserved *S. bacillaris* were not distinct in any of the CPAs. But, glycerol produced a lower growth than Me_2SO.

The effects of slow and rapid cooling on the growth of *C. minutus* and *S. nidulans* were not distinct in any of the CPAs. Maximum growth of *O. angustissima* was recorded with 33980.0 x 10^4 cells mL^{-1} in Me_2SO following slow cooling. *T. erythraeum* grew best in Me_2SO using the slow cooling method. *Tetraselmis* grew better in glycerol than in Me_2SO, but growth was very low less than 30.0 x 10^4 cells mL^{-1}.

The daily specific growth rate of each microalgal group by class in this experiment is shown in Table 4. The effects of slow and rapid cooling on the growth rate of all the cryopreserved Chlorophyceae and Cyanophyceae were not significantly different for Me_2SO and glycerol ($p < 0.05$). The growth rate of all the Eustigmatophyceae was the highest significantly in Me_2SO following the rapid cooling method ($p < 0.05$). Prasinophyceae grew only in glycerol, but freezing method did not affect the growth rate. In general, the effects of slow and rapid cooling on the growth rate of most cryopreserved marine microalgae were not different to each other and in all cases and Me_2SO proved a more effective growing medium than glycerol except Prasinophyceae.

Effect of cell concentrations in cryopreservation and removal of Me_2SO after thawing

In this experiment we used 10% Me_2SO as a CPA because most of the microalgae in the previous experi-

Table 3. Mean daily specific growth rates for fifteen-day cultures of each microalgal species cryopreserved with different cryoprotectant agents and cooling methods for one month (I and III, kept in -196°C directly; II and IV, kept in -196°C after pre-cooling (-1°C min^{-1}) to -80°C. No living cell was found on the final culture day. A different superscripted lower case letter in the same row means a significant difference at the $p < 0.05$ level)

Species	Me_2SO		Glycerol	
	I	II	III	IV
Gloeocystis gigas	0.22 ± 0.0345^a	0.24 ± 0.1256^a	0.13 ± 0.0778^a	0.15 ± 0.0906^a
Nannochloris oculata	0.02 ± 0.0783^a	-0.09 ± 0.0563^b	-0.13 ± 0.0578^b	-0.11 ± 0.0439^b
Stichococcus bacillaris	0.25 ± 0.0159^a	0.19 ± 0.0197^a	0.18 ± 0.0804^a	0.16 ± 0.1573^a
All Chlorophyceae	0.16 ± 0.1154^a	0.11 ± 0.1697^a	0.06 ± 0.1562^a	0.07 ± 0.1606^a
Oscillatoria angustissima	0.35 ± 0.299^a	0.36 ± 0.0319^a	-0.41 ± 0.0387^b	-0.37 ± 0.0^b
Chrococcus minutus	0.03 ± 0.0512^b	0.23 ± 0.1100^a	-0.24 ± 0.0405^c	-0.10 ± 0.1425^c
Synechococcus nidulans	0.18 ± 0.0575^b	0.24 ± 0.0276^a	0.00 ± 0.0213^c	0.00 ± 0.0333^c
Trichodesmium erythraeum	-0.04 ± 0.0997^b	0.11 ± 0.0954^a	-	-
All Cyanophyceae	0.13 ± 0.1652^a	0.23 ± 0.1128^a	-0.16 ± 0.1875^b	-0.07 ± 0.1397^b
Nannochloropsis oceanica	0.23 ± 0.0124^a	0.22 ± 0.0139^a	0.01 ± 0.0295^b	0.23 ± 0.0325^a
Nannochloropsis salina	0.17 ± 0.0189^a	0.18 ± 0.0187^a	0.00 ± 0.0241^b	-0.04 ± 0.0391^c
Nannochloropsis sp.	0.27 ± 0.0271^a	0.13 ± 0.1703^b	-0.03 ± 0.0354^c	-0.04 ± 0.0166^c
All Eustigmatophyceae	0.22 ± 0.0455^a	0.18 ± 0.1007^a	-0.01 ± 0.0326^b	0.05 ± 0.1313^b
Tetraselmis striata	-0.04 ± 0.0082^c	0.12 ± 0.0369^a	0.05 ± 0.0131^b	0.04 ± 0.0500^b
Tetraselmis suecica	0.00 ± 0.0142^c	0.00 ± 0.0106^c	0.04 ± 0.0193^b	0.05 ± 0.0063^a
All Prasinophyceae	-0.02 ± 0.0022^b	0.06 ± 0.0665^a	0.04 ± 0.0171^a	0.05 ± 0.0344^a
All microalgae	0.14 ± 0.1375^a	0.16 ± 0.1354^a	0.00 ± 0.1404^b	0.03 ± 0.1385^b

Table 4. Mean daily specific growth rate for fifteen-day culture of each microalgal species cryopreserved with different cryoprotectant agents and cooling methods for six months (I and III, kept in -196°C directly; II and IV, kept in -196°C after pre-cooling (-1°C min^{-1}) to -80°C. No living cell was found on the final culture day. A different superscripted lower case letter in the same row means a significant difference at the $p < 0.05$ level)

Species	Me_2SO		Glycerol	
	I	II	III	IV
Gloeocystis gigas	0.21 ± 0.0301^a	0.22 ± 0.1157^a	0.14 ± 0.0585^a	0.13 ± 0.0692^a
Nannochloris oculata	0.05 ± 0.0572^a	-0.08 ± 0.0416^b	-0.10 ± 0.0391^b	-0.12 ± 0.0572^b
Stichococcus bacillaris	0.23 ± 0.0157^a	0.20 ± 0.0228^a	0.17 ± 0.0582^{ab}	0.12 ± 0.0711^b
All Chlorophyceae	0.16 ± 0.0894^a	0.11 ± 0.1576^{ab}	0.07 ± 0.1365^b	0.05 ± 0.1344^b
Oscillatoria angustissima	0.36 ± 0.0273^a	0.37 ± 0.0240^a	-0.47 ± 0.1207^b	-0.55 ± 0.1028^b
Chrococcus minutus	0.01 ± 0.0375^a	0.09 ± 0.0726^a	-0.25 ± 0.0414^c	-0.13 ± 0.1038^b
Synechococcus nidulans	0.21 ± 0.0128^a	0.22 ± 0.0180^a	-0.01 ± 0.0232^b	0.00 ± 0.0333^b
Trichodesmium erythraeum	-0.03 ± 0.0911^b	0.12 ± 0.0874^a	-	-
All Cyanophyceae	0.13 ± 0.1667^a	0.20 ± 0.1241^a	-0.15 ± 0.1952^b	-0.09 ± 0.1280^b
Nannochloropsis oceanica	0.27 ± 0.0255^a	0.19 ± 0.0355^b	0.07 ± 0.0476^c	-0.06 ± 0.0139^d
Nannochloropsis salina	0.18 ± 0.0139^a	0.17 ± 0.0183^a	0.01 ± 0.0346^b	0.01 ± 0.0346^b
Nannochloropsis sp.	0.23 ± 0.0190^a	0.22 ± 0.0201^a	-0.02 ± 0.0157^b	-0.01 ± 0.0222^b
All Eustigmatophyceae	0.22 ± 0.0422^a	0.20 ± 0.0313^b	0.02 ± 0.0497^c	-0.34 ± 0.0337^d
Tetraselmis striata	-	-	-0.07 ± -0.0516^a	-0.03 ± -0.0290^a
Tetraselmis suecica	-	-	0.02 ± 0.1002^a	-0.00 ± -0.0215^b
All Prasinophyceae	-	-	0.20 ± 0.0557^a	0.30 ± 0.0374^a
All microalgae	0.17 ± 0.1266^a	0.17 ± 0.1237^a	-0.01 ± 0.1373^b	-0.20 ± 0.1080^b

ments were successfully cryopreserved using 10% Me_2SO. The result of the specific growth rate of 11 microalgal species is shown in Table 5. The growth rate of *C. gracilis* with the Me_2SO removed in a non-concentrated sample was significantly higher than that in a concentrated one ($p < 0.05$). *N. annexa* showed the highest

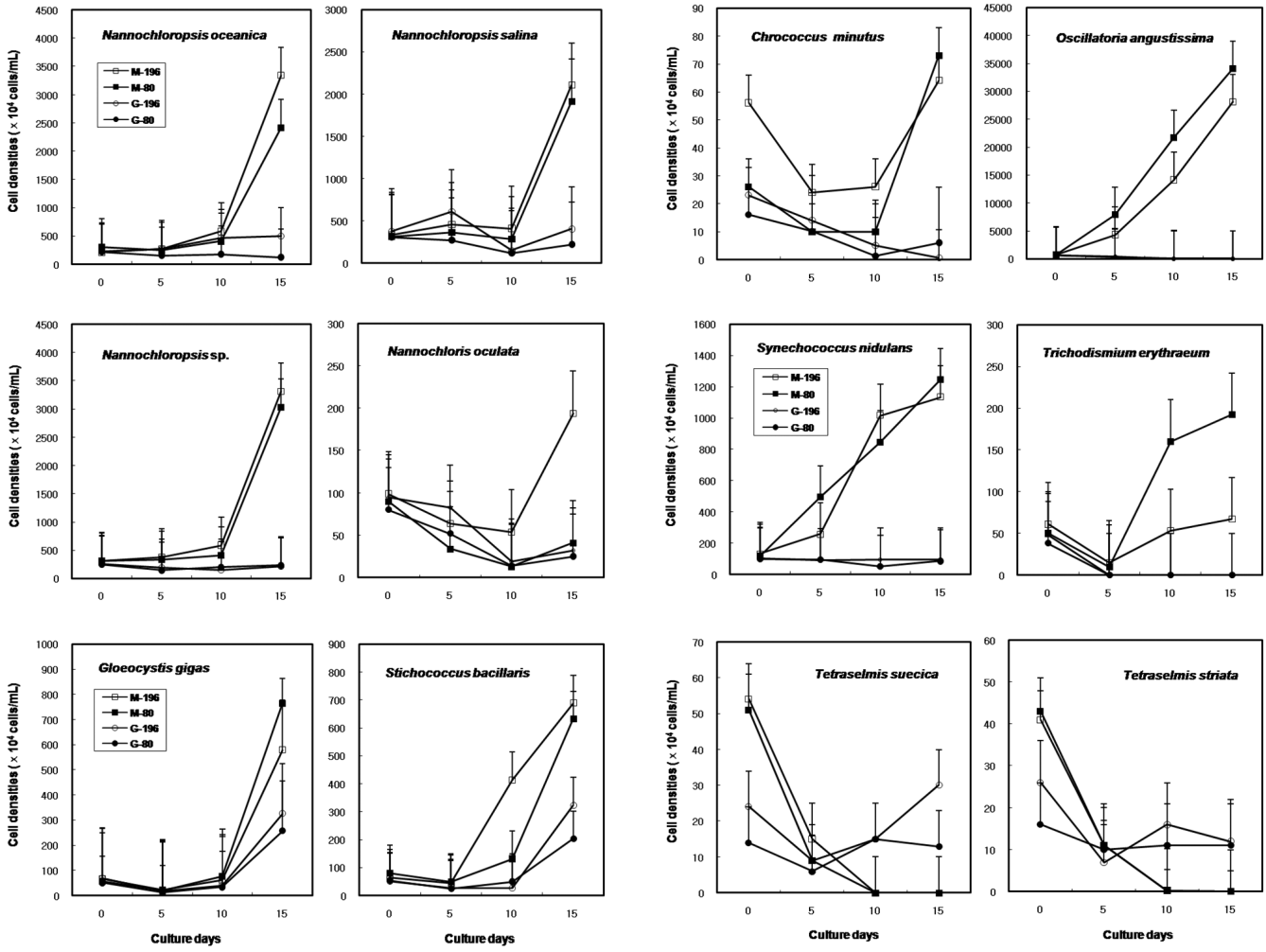

Fig. 2. Growth of each microalgal species cryopreserved with different cryopreservative agents and cooling methods for six months (M, Me_2SO; G, Glycerol; -196, kept in -196°C directly; -80, kept in -196°C after keeping in -20°C and -80°C for 10 min, respectively).

growth rate in a non-concentrated sample with the Me_2SO ($p < 0.05$). Removing 10% Me_2SO after thawing had no influence on the growth of the cells in a concentrated sample. *P. tricornutum* grew the best significantly in a non-concentration sample with the Me_2SO removed ($p < 0.05$). The growth rate of *S. costatum* was not influenced by the removal of the Me_2SO.

N. oculata grew better in a non-centrifugation sample regardless of whether the Me_2SO was removed or not. But *S. maxima* grew better with Me_2SO than without it regardless of the concentration. *N. oceanica* showed the highest growth rate significantly in a non-concentrated sample with the Me_2SO removed ($p < 0.05$). Of the *Tetraselmis*, *T. striata* grew better in a non-concentration sample with the Me_2SO in place. Only the growth rate of *T. suecica* was not influenced by cell concentration and the Me_2SO. *I. galbana* grew better in a non-concentrated sample than a concentrated one regardless of whether Me_2SO was present or not. But *P. pinguis*, grew better in a concentrated sample with the Me_2SO removed.

DISCUSSION

Freeze-drying is generally considered a better technique than serial transference, and the equipment required is minimal. Freeze-drying, however, is unreliable, often affording survival rates less than 5% (Mcgrath *et al.* 1978). Cryopreservation is a method that has significant potential for ensuring the long-term conservation and genetic stability of microalgal cultures (Poncet and Véron 2003). Many factors affect the effectiveness of cryopreservation in microalgal. But, one of the most important factors is the composition of the freezing medium. Although a good survival of deep-frozen microbes has

Table 5. Mean daily specific growth rate for two months of each microalgal species cryopreserved for one year with different cryopreservation concentrations and using the removal of Me_2SO after thawing culture method (I and III, non-removal of Me_2SO after thawing; II and IV, removal of Me_2SO after thawing. A different superscripted lower case letter in the same row means a significant difference at the $p < 0.05$ level)

	Species	Non-concentration		Concentration	
		I	II	III	IV
Bacillariophyceae	*Chaetoceros gracilis*	0.04 ± 0.0502^{ab}	0.07 ± 0.0269^{a}	0.04 ± 0.0354^{ab}	0.01 ± 0.0158^{b}
	Navicula annexa	0.19 ± 0.0339^{a}	0.16 ± 0.0298^{b}	0.09 ± 0.0202^{c}	0.09 ± 0.0520^{c}
	Phaeodactylum tricornutum	0.13 ± 0.0067^{b}	0.14 ± 0.0181^{a}	0.08 ± 0.0067^{c}	0.05 ± 0.0255^{d}
	Skeletonema costatum	0.02 ± 0.0375^{b}	0.05 ± 0.0445^{ab}	0.07 ± 0.0423^{ab}	0.08 ± 0.0666^{a}
Chlorophyceae	*Nannochloris oculata*	0.14 ± 0.0083^{a}	0.14 ± 0.0115^{a}	0.10 ± 0.0061^{b}	0.09 ± 0.0078^{c}
Cyanophyceae	*Spirulina maxima*	0.18 ± 0.0046^{a}	0.16 ± 0.0138^{b}	0.17 ± 0.0122^{a}	0.16 ± 0.0099^{b}
Eustigmatophyceae	*Nannochloropsis oceanica*	0.12 ± 0.0064^{b}	0.13 ± 0.0151^{a}	0.09 ± 0.0101^{c}	0.09 ± 0.0068^{c}
Prasinophyceae	*Tetraselmis striata*	0.18 ± 0.0360^{a}	0.04 ± 0.0392^{b}	0.08 ± 0.0546^{b}	0.08 ± 0.0481^{b}
	Tetraselmis suecica	0.03 ± 0.0421^{a}	0.04 ± 0.0530^{a}	0.02 ± 0.0192^{a}	0.02 ± 0.0162^{a}
Prymnesiophyceae	*Isochrysis galbana*	0.20 ± 0.0354^{a}	0.21 ± 0.0264^{a}	0.15 ± 0.0268^{b}	0.15 ± 0.0208^{b}
	Pavlova pinguis	0.10 ± 0.0413^{bc}	0.07 ± 0.0258^{c}	0.12 ± 0.0335^{b}	0.17 ± 0.0243^{a}

occasionally been observed without a protective additive, the presence of a suitable CPA usually increases the survival considerably (Hubálek 2003). The most commonly used cryoprotectants in microalgae cryopreservation include: Me_2SO, glycerol, methanol (MeOH), proline, polyvinylpyrrolidone and sorbitol (Cañavate and Lubian 1995a). However, CPAs are cytotoxic more or less. It is necessary to determine the tolerance of cells to these compounds, especially when using the compounds that penetrate the cells such as glycerol and Me_2SO. Fenwick and Day (1992) reported the effect of different CPAs on cell motility in *Tetraselmis suecica* and glycerol and Me_2SO were the least toxic. Prolonged exposure to these cryoprotectants can actually harm the cells, so equilibration time and concentration of the cryoprotectant are important factors to control.

Cryoprotectant concentrations vary from 1 to 30% (v/v) in the alga culture media, but 5-10% solutions are most common. However, survival of cryopreserved algae largely depends on the degree of damage incurred during the freezing and thawing process (Fenwick and Day 1992). Houdan *et al.* (2005) demonstrates that Me_2SO (7.5%) could efficiently be used as a cryoprotectant for both stages of the coccolithophore Emiliania huxleyi if subjected to a controlled rate of cooling. Haptophytes *Chrysochromulina simplex*, *Prymnesium parvum*, and *P. parvum* f. *patelliferum* survived in a cryopreservation solution of 15% Me_2SO, as did three dinoglagellates: *Amphidinium carterae*, *A. trulla*, and *Gymnodinium simples* (Rhodes *et al.* 2006).

There have been ongoing efforts to improve freezing methods. Morris (1976, 1978) examines the viability of 250 strains of cryopreserved *Chlorococcales*; and Ben-Amotz and Gilboa (1980a, 1980b) tests 12 strains of marine green algae. In general, the cooling method consists of slowly cooling the cultures at the rate of about -1°C min^{-1} between - 25°C to -70°C, and then plunging them into liquid nitrogen for storage (Ben-Amotz and Gilboa 1980a, 1980b; Day *et al.* 1997; Taylor and Fletcher 1999; Tzovenis *et al.* 2004).

In the cryopreservation experiment on Navicula subinflata by Redekar and Wagh (2000), the optimum concentration and exposure times of ethylene glycol, Me_2SO and methanol are 4 M and 30 minute, respectively. After cryopreservation for 0 day, 7 days and 30 days, the maximum microalgae growth rate was recorded for ethylene glycol but there was no growth in methanol.

Tetraselmis was successfully cryopreserved with 10 or 15% Me_2SO (Rhodes *et al.* 2006). According to Fenwick and Day (Fenwick and Day 1992), *Tetraselmis* is better cryopreserved with glycerol than with Me_2SO. For *T. suecica* (Fenwick and Day 1992; Montaini *et al.* 1995) glycerol had the most protective effect with post-thaw viability levels of over 70%. *T. chuii* was cooled at a rate of 0.5°C min^{-1} from 0°C to -50°C before being plunged into liquid nitrogen. *T. chuii* was successfully cryopreserved using 5% Me_2SO for 3-5 weeks (Cañavate and Lubian 1997a). In this study, *T. suecica* was also successfully cryopreserved with rapid cooling using 10% glycerol.

Nannochloris oculata displayed different levels of post-thaw viability depending on the CPAs (Poncet and Véron 2003). Microalgae cryopreserved with methanol

did not grow for the first six days. In contrast, that of the unfrozen methanol-treated control cultures increased over this period. After six days in culture, the growth of the cryopreserved microalgae subsequently recovered, but cell density at the end of the experiment was lower than that of the control group. Gwo *et al.* (2005) finds that the viability of *N. oculata* is best when using a cooling rate of 1°C min^{-1} to -40°C and liquid nitrogen with the addition of either Me_2SO or propylene glycol at a 10 or 20% concentration. *N. oculata* was successfully cryopreserved using Me_2SO 2.5 M and 2.2 M glycerol. The viability rate of *N. gaditana*, which was cryoperseved with 5% methanol for seven days, was 49% (Cañavate and Lubian 1995b). The cryopreservation of this microalgal species at the lowest cooling rate (0.5°C min^{-1}) in a medium of 20 ppt salinity showed higher viability regardless of whether Me_2SO was added or not (Cañavate and Lubian 1997a).

After being frozen using a rapid uncontrolled cooling method - a mechanical freezer set at -60°C, - bloom-forming cyanobacterial strains such as *Microcystis, Oscillatoria, Anabaena*, and *Aphanizomenon* grew sufficiently for the test of post-thaw viability. Complete viability of all the tested strains was found after 2.5 years of cryopreservation without cryoprotectant (Park 2006).

Many previous studies have considered Me_2SO as one of the best CPAs for microalgae (Taylor and Fletcher 1999). Me_2SO has been used successfully to preserve microalgae at concentrations ranging from 3% v/v for the freshwater cyanobacterium *Microcystis aeruginosa* to 15% for several marine species belonging to different Phyla (Cañavate and Lubian 1995a). However, in this study, *O. angustissima* was successfully cryopreserved using 10% Me_2SO. The effect of slow and rapid cooling on the viability of *O. angustissima* was different in Me_2SO.

On the other hand, new technique on encapsulation and dehydration of cryopreserved cell has recently developed to reduce the mortality of the microalgae due to freezing. However, the results of this technique up to now are also variable according to microalgal species (Zhang *et al.* 2009). Using this technique, the viability of *Nannochloropsis* sp., *Nostoc commune* and *Phormidium foveolarum* were successfully recovered after storage in liquid nitrogen. But that of *Spirulina subsalsa*, *Volvox aureus*, *Pandorina morum* and *Rhodomonas ovalis* did not grow (Hirata *et al.* 1996; Day *et al.* 2000).

Many results on microalgae cryopreservation techniques have been reported. Nevertheless, only a fraction of the large number of microalgae kept in culture collections have been successfully regenerated after storage in liquid nitrogen (15% of the National institute for Environmental Studies in Japan, 35% of Culture Collection of Algae and Protozoa in the United Kingdom (Day *et al.* 1998), and 40% of the Provasoli-Guillard National Center for Culture of Marine Phytoplankton in the U.S.A, ccmp.bigelow.org, 2009).

In this study, microalgal species with thick cell walls without flagellates or seta were successfully cryopreserved. When compared with glycerol, Me_2SO proved the most efficacious for cryopreservation. The cell concentration and amount of time in cryopreservation had no affect on the viability and growth of the cell. In addition, growth of microalgae after thawing was not influenced by the removal of the 10% Me_2SO. The age of a cell culture was also considered as an important factor in determining its viability. The resistance of cells to the stress of freezing and thawing increases at the stationary phase of growth because cells in this physiological state accumulate lipid content (Harding *et al.* 2004). The viability and growth of cryopreserved microalgae depends on the species. There is unlikely to be a uniform protocol that could be applied to various circumstances. The use of cryopreservation at the KMMCC is still at a preliminary phase of investigation, although a significant number of microalgal species has been successfully cryopreserved. It is difficult to infer the best cryopreservation method for microalgal species. Cryopreservation affects different species differently. Therefore, the optimum condition for successful cryopreservation should be examined for each microalgal species.

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