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Long-term Preservation of Bloom-forming Cyanobacteria by Cryopreservation

Hae-Kyung Park

Han River Environment Research Center, National Institute of Environmental Research, 627, Yangsu-ri, Yangseo-myon, Yangpyung-gun, Kyunggi-do 476-823, Korea

Long-term preservation of bloom-forming cyanobacteria was evaluated using cryopreservation and freeze-drying of nine strains belonging to four genera and seven species. All test strains, except *Aphanizomenon flos-aquae* NIER-10028, showed partial or complete survival following cryopreservation and freeze-drying. Frozen and freeze-dried strains were preserved for more than two years and were revived monthly. Most strains showed higher post-thaw viability after cryopreservation, especially without cryoprotectant compared to freeze-drying. *Microcystis aeruginosa* NIER-10010, *M. viridis* NIER-10020, *M. ichthyoblabe* NIER-10023, *M. novacekii* NIER-10029 and *Oscillatoria sancta* NIER-10027 were revived after 2.5 years of cryopreservation. These results suggest that cryopreservation may be an easy and timesaving long-term preservation method for bloom-forming cyanobacteria.

Key Words: cryopreservation, cyanobacteria, freeze-drying, *Microcystis*, *Oscillatoria*

INTRODUCTION

In many temperate and tropic fresh waters, a few cyanobacterial genera, including *Microcystis*, *Anabaena*, *Oscillatoria*, *Aphanizomenon*, *Phormidium*, often dominate and induce water blooms resulting in detrimental changes in the water quality in addition to acquiring some undesirable impacts to water utilization, such as, clogging the sand filters, production of distinctive taste and odor compounds, and health hazard problems (Wicks and Thiel 1990; Carmichael and Saffermann 1992; Park *et al*. 2000). Therefore there has been a high demand for bloom-forming cyanobacterial strains by both the academic and industrial worlds. However, unlike the culture collection of bacteria, fungi, organisms which have been actively studied for a long time, the isolation, cultivation, and preservation of bloom-forming cyanobacteria are technically much more complex.

Various methods have been used to preserve algae, including serial transfer, freeze-drying, and cryopreservation (Andersen 1996; Day *et al*. 1997). However by far, the most widely used method for maintenance of algal strains, especially bloom-forming cyanobacteria is by serial transfer (Hosaka 1994; Andersen 1996). Serial transfer of cyanobacterial strains requires no expensive equipment and is generally very satisfactory for the maintenance of a small number of noncritical cultures. But it is a labor-intensive, time-consuming process with a possibility of contamination and genetic drift (Apt and Behrens 1999). Cryopreservation is a process that involves freezing and storage of cells at a very low temperature. It is generally regarded as the single best method for the long-term preservation of organisms and their properties (Heckly 1978; Ashwood-Smith and Farrant 1980; Andersen 1996; Apt and Behrens 1999; Gorman and Adley 2004). Although some degrees of success with cryopreservation techniques have been reported with several different algal groups including green algae, red algae, euglenophytes, diatoms, and cyanobacteria (Morris 1978; Simione and Brown 1991; Watanabe *et al*. 1992; Hosaka 1994; Canavate and Lubian 1997; Day *et al*. 1997), many strains are apparently freeze-recalcitrant (Day *et al*. 1998).

In this study, long-term preservation of bloom-forming cyanobacteria was evaluated using cryopreservation and freeze-drying of nine strains belonging to four genera and seven species.

MATERIALS AND METHODS

Cyanobacterial strains

Nine strains of bloom-forming cyanobacteria belonging to four genera and seven species were used for the long-term preservation (Table 1). These strains had been

*Corresponding author (parkhk@me.go.kr)

Table 1. List of test cyanobacterial strains

Scientific name	Collection date	State in liquid medium
Microcystis aeruginosa NIER-10010	Jun. 1993	Colony form
Microcystis aeruginosa NIER-10024	Sep. 1996	Suspended form
Microcystis ichthyoblabe NIER-10021	Sep. 1996.	Colony form
Microcystis ichthyoblabe NIER-10023	Sep. 1996	Colony form
Microcystis viridis NIER-10020	Sep. 1996	Colony form
Microcystis novacekii NIER-10029	Sep. 1995	Colony form
Anabaena macrospora NIER-10016	Sep. 1995	Straight trichome
Oscillatoria sancta NIER-10027	Oct. 1996	Straight trichome
Aphanizomenon flos-aquae NIER-10028	Oct. 1996	Trichomes in bundle form

isolated from the Korean lakes and rivers. All strains have been maintained at the NIER (National Institute of Environmental Research of Korea) Culture Collection by serial transfer after isolation and in uni-algal states. Among six strains of *Microcystis* genus, five strains (*Microcystis aeruginosa* NIER-10010, *Microcystis viridis* NIER-10020, *Microcystis ichthyoblabe* NIER-10021, *M. ichthyoblabe* NIER-10023, *Microcystis novacekii* NIER-10029) form distinctive colonies in liquid media.

Sample preparation for cryopreservation

Cyanobacterial strains were cultured in 200 ml CB liquid media (Watanabe and Nozaki 1994) at 23°C, 2000 lux, 16hrs. light/8 hrs. dark condition, harvested in the late logarithmic phase of growth, and then concentrated to 10^3-10^4 cells/ml by settling.

To evaluate the effect of the cryoprotectant, samples were prepared in two conditions, with or without cryoprotectant. Dimethyl sulfoxide (DMSO) was used as a cryoprotectant in this study. DMSO was added at 5% concentration level. Filamentous cyanobacterial strains (*Anabaena macrospora* NIER-10016, *Oscillatoria sancta* NIER-10027, *Aphanizomenon flos-aquae* NIER-10028) were cryopreserved only without cryoprotectant.

Each milliliter of concentrated cell suspension was transferred into each of 1.8 milliliter cryogenic tube (Nunc cryotubes), placed into the bottom of mechanical freezer (Revco Ultima II ULT 2186 9D) set at -60°C directly, and stored until the recovery test was carried out. One milliliter of the untreated cell suspension was immediately inoculated into a new CB medium to confirm the viability of pre-cultures as a control.

Sample preparation for freeze-drying

One milliliter of concentrated cell suspension was transferred into 2 milliliter glass ampules and placed into the -60°C mechanical freezer. Frozen suspension was immediately transferred to the freeze-dryer (Labconco Freezone 77590) and at the end of the drying process, glass ampules were sealed with a double-flame air/gas torch and preserved in the -60°C mechanical freezer.

Recovery test

Five tubes or ampules of each strain were revived immediately after freezing and freeze-drying to examine the post-thaw viability after each treatment. The rest of the frozen and freeze-dried samples were revived every month for more than two years. Five tubes or ampules of each strain were revived at each term. Frozen samples were thawed rapidly by placing the tubes in a water bath set at 40°C (Watanabe *et al.* 1984) until the ice had melted. The thawed suspension was then transferred directly into the test tube containing 10 milliliter of fresh CB liquid media and cultured under the same condition of preculture. For the freeze-dried sample, an aliquot of fresh CB liquid media was added into the ampule and a rehydrated pellet was transferred into the tube containing fresh medium. The growth state of each tube had been observed everyday after inoculation for more than one month. Viability of frozen or freeze-dried sample was evaluated as follows: "+" if it showed full growth after inoculation as seen in the control culture, and "±" if it revived but did not reach its full growth compared with the control culture, and "–" if it did not show any signs of growth for a month or longer.

RESULTS

The viability and the lag phase periods of test strains just after freezing and freeze-drying treatment are shown in Table 2. Among the test strains, only *Aph. flos-aquae* NIER-10028 did not revive after freezing and freeze-drying treatment while the rests of strains revived partially or completely. Most of the strains showed a higher post-

Table 2. The post-thaw viability and the period of lag phase of test strains just after freeze-drying and freezing treatment (NT: not tested, +: full growth after inoculation as seen in the control culture, ±: revived but did not reach its full growth compared with the control culture, – (recovery): no sign of growth for a month or longer)

NIER No.	Freeze-drying		Cryopreservation (without DMSO)		Cryopreservation (with DMSO)	
	Viability (%)	Lag phase (day)	Viability (%)	Lag phase (day)	Viability (%)	Lag phase (day)
10010	+++±±(80)	19-24	+++++(100)	15-21	+++±±(80)	20-22
10024	−−−−−(0)	-	+++++(100)	17-22	+++++(100)	13-15
10021	±±−−−(20)	19-29	+++++(100)	8-15	+++++(100)	13-22
10023	+−−−−(20)	30	+++++(100)	11-14	++++−(80)	13-18
10020	−−−−−(0)	-	+++++(100)	5-7	++++±(90)	19-25
10029	−−−−−(0)	-	+++++(100)	5-7	+++++(100)	17-19
10016	−−−−−(0)	-	+++±±(80)	17-25	NT	NT
10027	+++−−(60)	19-22	+++++(100)	4-6	NT	NT
10028	−−−−−	-	−−−−−	-	NT	NT

Table 3. The post-thaw viability of test strains according to the preservation time for one year preservation (NT: not tested, FD: freeze-drying, C (-) : cryopreservation with DMSO, C (+): Cryopreservation without DMSO, +: full growth after inoculation as seen in the control culture, ±: revived but did not reach its full growth compared with the control culture, –: no sign of growth for a month or longer)

Strain	Month	Viability (% recovery)					
		1	3	5	7	9	12
NIER-10010	FD	+±±±−(50)	+++±±(80)	+±±±±(60)	++±−−(50)	+++±−(70)	++++±(90)
	C(-D)	+++±±(80)	+++±±(80)	+++++(100)	++++±(90)	+++++(100)	++++±(90)
	C(+D)	+±±±±(60)	+±±±±(60)	±±±±−(40)	±±±±±(50)	±±±±±(50)	++±±−(60)
NIER-10020	FD	NT	+−−−−(20)	++−−−(40)	NT	−−−−−(0)	+±±−−(40)
	C(-D)	+++++(100)	+++++(100)	+++++(100)	++++−(80)	+++++(100)	+++++(100)
	C(+D)	++±±−(60)	±±−−−(20)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)
NIER-10021	FD	++++−(80)	++−−−(40)	++−−−(40)	+++++(100)	+++++(100)	NT
	C(-D)	+++++(100)	+++++(100)	NT	++++−(80)	+++++(100)	+++++(100)
	C(+D)	+++++	+++−−(60)	NT	+++++(100)	+++++(100)	+++++(100)
NIER-10023	FD	−−−−−(0)	−−−−−(0)	−−−−−(0)	++−−−(40)	−−−−−(0)	−−−−−(0)
	C(-D)	+++++(100)	+++++(100)	+++++(100)	+++++(100)	+++++(100)	+++++(100)
	C(+D)	+++++(100)	++++−(80)	+++−−(60)	++±±−(60)	+−−−−(20)	+−−−−(20)
NIER-10024	FD	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)
	C(-D)	++++±(90)	++++−(80)	+++++(100)	NT	++++−(80)	+++±−(70)
	C(+D)	+++++(100)	++±±±(70)	++++±(90)	NT	++++−(80)	+++++(100)
NIER-10029	FD	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	NT
	C(-D)	+++++(100)	+++++(100)	+++++(100)	+++++(100)	++++−(80)	+++++(100)
	C(+D)	+++++(100)	+++−−(60)	+++++(100)	++++±(90)	+±±±−(50)	+±±±−(50)
NIER-10016	FD	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)
	C	++++−	+++++(100)	+++−−(60)	++++±(90)	++±−−(50)	+++±−(70)
NIER-10027	FD	+++++(100)	+++++(100)	+++−−(60	+++++(100)	+++++(100)	++−−−(40)
	C	+++++(100)	+++++(100)	+++++(100)	+++++(100)	+++++(100)	+++++(100)
NIER-10028	FD	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)
	C	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)	−−−−−(0)

thaw viability after freezing compared to freeze-drying.

All strains which were frozen without cryoprotectant showed a higher post-thaw viability compared to freezing with cryoprotectant. All samples of *M. ichthyoblabe* NIER-10021, *M. aeruginosa* NIER-10024 and *M. novacekii* NIER-10029 showed full growth when compared with

controls which were cultured by serial transfer, regardless of addition of cryoprotectant. Whereas samples which cryopreserved with cryoprotectant needed a longer lag phase.

There were significant differences in the period of lag phase, known as the period of growth initiation, after inoculation depending on the preservation methods. By the serial transfer, all the test strains started to grow without a lag phase and showed a maximum growth within 10 days. However, as shown in Table 2, most of frozen or freeze-dried samples started to grow after 4 to 30 days of the lag phase. This result implies that strains need a lag phase to recover from cell damages by the preservation process. In addition, the strain which showed the shorter lag phase, showed the higher post-thaw viability, implying that the extent of cell damage could affect the length of the lag phase period.

The viability level after one-year preservation is shown in Table 3. The post-thaw viability varied depending on the preservation methods and strains. *M. aeruginosa* NIER-10010, *M. viridis* NIER-10020, *M. ichthyoblabe* NIER-10021, *M. ichthyoblabe* NIER-10023, *M. novacekii* NIER-10029, *A. macrospora* NIER-10016, *Oscil. sancta* NIER-10027 revived completely after one year of cryopreservation without cryoprotectant. Only *M. aeruginosa* NIER-10010 revived after one year of freeze-drying. There were no significant differences in the viability level depending on the preservation period.

There was a gradual decline in the viability depending on the cryopreservation period in several strains which were cryopreserved with cryoprotectant. *M. ichthyoblabe* NIER-10023, which was cryopreserved with cryoprotectant, revived completely after two months, but viability level started to decline after three months and finally only 20% of viability was shown after one year preservation.

Several strains were revived after 2.5-year of long-term cryopreservation (Table 4). *M. viridis* NIER-10020, *M. ichthyoblabe* NIER-10023, *M. novacekii* NIER-10029, *Oscil. sancta* NIER-10026, which were cryopreserved without cryoprotectant, revived completely.

The change of microscopic morphology was observed during the recovery process. When the cryopreserved *M. aeruginosa* NIER-10010 forming firm colonies were inoculated into the culture media after thawing, green colonies were observed with the naked eye in the water surface but distinct cell shape was not observed microscopically just after the inoculation (Fig. 1A). Green colonies changed into white colonies after a few days of culture and precipitated on the bottom of the test tube during the lag phase (Fig. 1, B). New green spots were revealed within white colonies as culturing days went by (Fig. 1, C) and finally small cell clusters changed to large new colonies (Fig. 1, D). This result implies that post-thaw viability might be the result of the growth of several surviving cells among dead cells at the inoculation time and not by the viability of all cells during the long lag phase.

Table 4. The post-thaw viability of test strains after 2.5-year cryopreservation (NT: not tested, +: full growth after inoculation as seen in the control culture, ±: revived but did not reach its full growth compared with the control culture, –: no sign of growth for a month or longer)

Scientific name	Condition	viability (% recovery)
Microcystis aeruginosa NIER-10010	with DMSO	+++±–±(70)
Microycstis viridis NIER-10020	without DMSO	++++±(100)
Microcystis ichthyoblabe NIER-10023	with DMSO	−−−−±(0)
	without DMSO	++++±(100)
Microcystis aeruginosa NIER-10024	with DMSO	+−−−±(20)
	without DMSO	++−−−±(40)
Microcystis novacekii NIER-10029	without DMSO	++++±(100)
Oscillatoria sancta NIER-10027	without DMSO	++++±(100)

DISCUSSIONS

From the results, bloom-forming cyanobacterial strains seemed to be more sensitive to freeze-drying than cryopreservation. Freeze-drying is generally considered a better technique than serial transfer, and the equipment required is minimal. But freeze-drying is unreliable, often giving survival rates of less than 5% (McGrath *et al.* 1978). It was reported that in general, akinete and heterocyst showed a higher level of viability from a long-term preservation than the vegetative cells (Watanabe *et al.* 1984). However, the present study showed different results, that is, *Oscillatoria* sp. and *Microcystis* spp. which exist only as vegetative cells showed higher post-thaw viability than *Aph. flos-aquae* NIER-10028 and *A. macrospora* NIER-10016 which have akinetes within trichomes, particularly following freeze-drying.

Several parameters are generally considered very important in cryopreservation, including the choice of the cryoprotectant, cryoprotectant concentration, freezing rate, physiological status of the culture and thawing

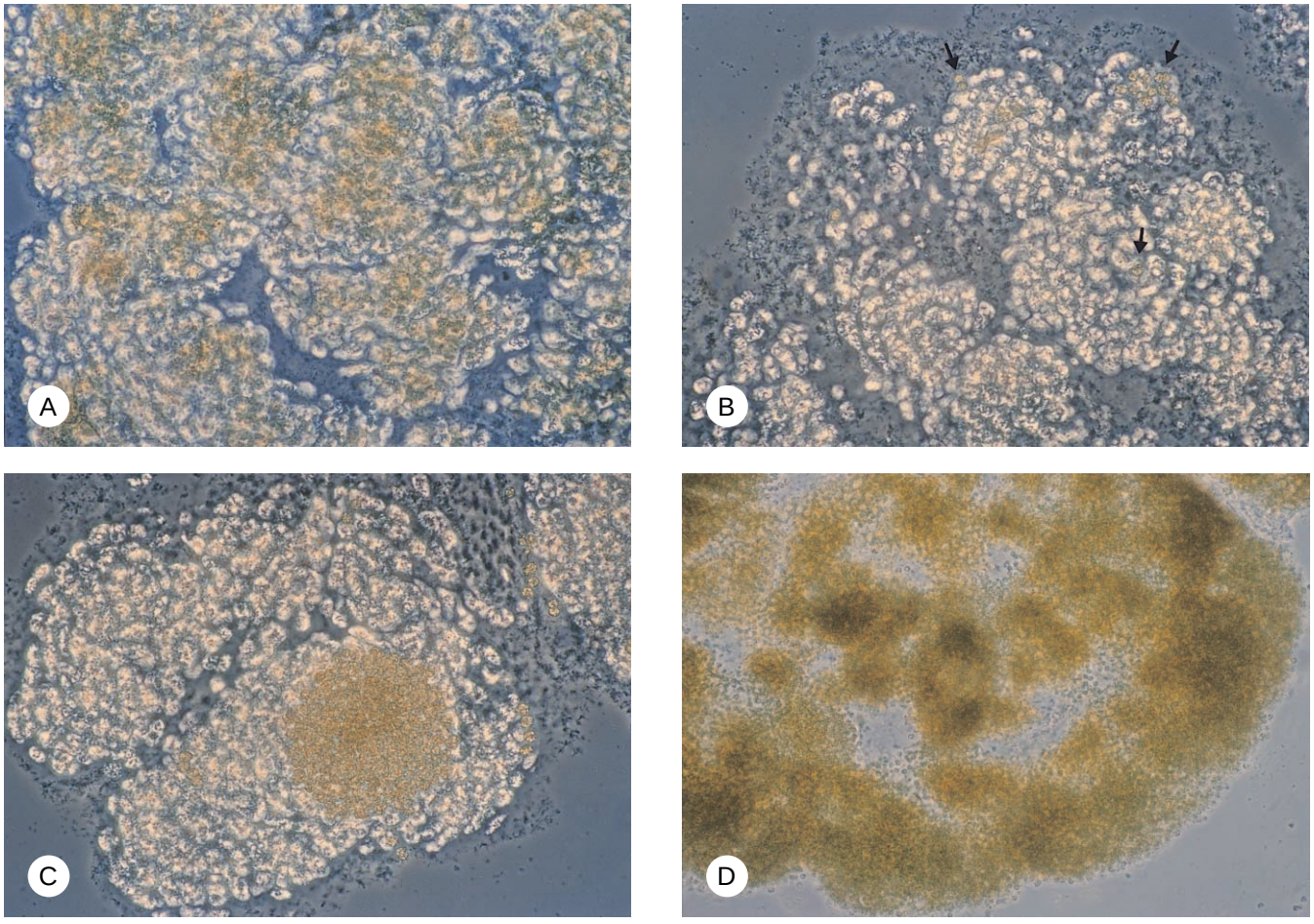

Fig. 1. Microphotographs of *Microcystis aeruginosa* NIER-10010 during the post-thaw revival process after cryopreservation; (A) green colonies at inoculation time, (B) white colonies during the lag phase (arrows point to the revived cells), (C) revived cells within white colonies, (D) mature new colonies.

procedure (Apt and Behrens 1999). Freezing a suspension of living cells resulted in several events that can be detrimental to the viability of these cells (Mazur 1977). A primary mode of lethal cell injury is the formation of intracellular ice (Day *et al.* 1998). Chemical compounds called cryoprotectant are added to the suspension of cells to help minimize the damage experienced during freezing. A wide variety of cryoprotectants have been tried, including DMSO, glycerol, methanol, polyvinylpyrrolidone, proline, propylene glycol, ethylene glycol, sorbitol, glucose, sucrose, dextran and betaine (Canavate and Lubian 1995; Andersen 1996; Kono *et al.* 1997). Glycerol, DMSO and methanol are the most widely used cryoprotectants and each has been shown to give good success rates (Beaty and Parker 1990; Canavate and Lubian 1995).

Skim milk, bovine serum albumin(BSA), and glycerol, which are often used as cryoprotectant for bacterial preservation, are not effective for cyanobacteria. Likewise, DMSO was known as the most effective cryoprotectant for algae (Simione and Brown 1991). However DMSO did not act so much as a cryoprotectant but as an inhibitor in this study. Watanabe *et al.* (1984) reported that a cryoprotectant was effective on improving the viability of coccoidal cyanobacteria. But then it was found to be ineffective at all and even inhibited the growth of filamentous cyanobacteria. But the cryoprotectant was not effective on the cryopreservation of coccoidal cyanobacteria, *Microcystis* strains in this study. Cryoprotectant toxicity may be one factor that is responsible for the cell damage on freezing and thawing, as the cells remain exposed to cryoprotectant during much of the freezing and thawing processes (Day *et al.* 1998).

To examine the changes of cell characteristics by cryopreservation, the patterns of the growth in liquid media after preservation treatment (i.e. buoyancy of cells and the colony shape), which are major classification keys of *Microcystis* species, were compared with control cultures.

During the latter stage of culture, the revived strains exhibited the same shapes of colonies and the same patterns of the growth in liquid media as seen in control cultures, suggesting little effects of cryopreservation on colony characteristics.

In order to minimize cold shock, controlled cooling rate in which 1°C per minute from room temperature to -40°C, then plunging into liquid nitrogen is generally preferred (Tsuru 1973; Canavate and Lubian 1995; Day *et al*. 1997). However, in this study, all the test strains, except *Aph. flos-aquae* NIER-10028, after being frozen using a rapid uncontrolled cooling method by a mechanical freezer set at -60°C, yielded for sufficiently successful levels of post-thaw viability. There were no significant differences in the level of post-thaw viability depending on the period of preservation. Complete viability was shown after 2.5 years of cryopreservation without cryoprotectant in most strains.

Many laboratories have isolated the cyanobacterial strains for the related studies and lost most of them after the studies were finished because of several factors like lack of proper preservation method, manpower and equipments such as a controlled cooling apparatus and liquid nitrogen chamber. This has resulted to a wasted effort of isolation and loss of research materials.

In this study, the typical bloom-forming cyanobacteria, *Microcystis*, *Anabaena*, *Oscillatoria* strains, which have been known to be difficult to apply with the long-term preservation, exhibited high post-thaw viability after more than two years cryopreservation. This result suggests that cryopreservation may be an easy and timesaving long-term preservation method for bloom-forming cyanobacteria not only in the algal culture collections but also in common laboratories.

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