

Cryopreservation of Microalgae and Cyanobacteria

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Summary

Most culturable cyanobacteria and soil microalgae can be cryopreserved with relatively high viability. Furthermore, many freshwater and marine eukaryotic algae can also be cryopreserved, but typically with lower post-thaw viability levels. However, to date, most dinoflagellates, cryptophytes, synurophytes, and raphidophytes cannot be successfully cryopreserved. Marine diatoms can be cryopreserved, and often have high viability, although freshwater diatoms have thus far proven more problematic. Large numbers of strains have been examined, most notably at the four major protistan collections: Culture Collection of Algae and Protozoa (CCAP) (UK), The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) (USA), Sammlung von Algenku Huren Göttingen (SAG) (Germany), and The Culture Collection of Algae at the University of Texas at Austin (UTEX) (USA), and it has been observed that chlorarachniophytes, eustigmatophytes, pelagophytes, phaeothamniophytes, and ulvophytes also have very high success rates, comparable with the other green algae and cyanobacteria. It has been noted that virtually all algae with a large cell size, as well as most filamentous strains, cannot as yet be cryopreserved. There are no known fundamental reasons why large and more complex algae cannot be successfully cryopreserved. Thus, it is anticipated that further research on the basic mechanisms of freezing damage and the empirical development of improved protocols will continue to expand the number and diversity of algal taxa that can be successfully cryopreserved.

Key Words: Algal cryopreservation; cryoinjury; cryostorage; cyanobacteria freezing; microalgal storage; culture collection.

1. Introduction

Unlike most other groups of microorganisms, prokaryotic cyanobacteria (blue-green algae) and eukaryotic microalgae have traditionally been maintained by routine serial subculture, with the frequency of transfer being largely determined by the growth characteristics of the strain. However, continuous maintenance of actively growing algal and cyanobacterial strains over long periods of time is relatively complex, time consuming, and, when it involves

From: *Methods in Molecular Biology*, vol. 368: *Cryopreservation and Freeze-Drying Protocols*, Second Edition
Edited by: J. G. Day and G. N. Stacey © Humana Press Inc., Totowa, NJ

large numbers of cultures, costly (1). In contrast, where cultures are kept “alive” in an arrested or retarded metabolic state they generally require a minimum of attention. Resting spores or other dormant stages of some species can be maintained at ambient or cool temperatures for many years without attention. For example, *Haematococcus pluvialis* aplanospores were recovered from air-dried soil after 27 yr (2), and the cyanobacterium *Nostoc commune* was revived from herbarium specimens after 107 yr of storage (3). However, the viabilities of resting stages generally decrease with time, and many aquatic algae do not have any dormant/resting stages. Freeze-drying has not been found to be a successful biostorage method for microalgae, with very low levels of viability (<1% of original population) (4) and a further reduction in viability on prolonged storage (5,6). The successful lyophilization of the cyanobacterium *Nostoc muscorum*, using a method similar to that detailed in Chapter 6, has been reported, with no observed reduction in viability after 5-yr storage (7). This technique has been adopted by a small number of researchers to preserve selected cyanobacterial strains. Where appropriate, effective methodologies have been developed; cryopreservation allows living algae that do not have any normal resting stage to be maintained indefinitely in an arrested state. High levels of viability are required to minimize the possibility of selecting an unrepresentative freeze-tolerant subpopulation. Furthermore, the relatively slow growth rate of algae (generation times in the range of 8 to 72 h are not uncommon), along with other problems associated with low viable cell density (see Note 1), can result in practical difficulties in reestablishing a viable culture.

Cryopreservation is employed at most of the major algal collections, and protocols adapted or developed empirically at these facilities typically employ relatively simple procedures that cryopreserve a broad range of algal species. The greatest degree of success has been achieved on cryopreserving cyanobacteria, marine pennate diatoms, and unicellular green algae, most of which are small and not morphologically complex (8). The methods described in this chapter have been applied successfully to a broad range of microalgae, including over 1400 strains in the CCAP and approx 1300 strains at UTEX (8). These procedures can be categorized as two-step freezing protocols. Two-step protocols require addition of a cell-permeating cryoprotectant to the algal culture, then the culture is cooled to a specified subzero temperature (step 1) to facilitate dehydration/cryodehydration of the sample. Next, it is cooled rapidly to the final storage temperature (step 2). The culture can be maintained at the storage temperature for an indefinite period of time. The cooling devices employed for algal cryopreservation, as for other cell types (see Chapters 5, 7, 9–23) generally fall into two categories: passive freezing systems and controlled cooling rate freezers. Three standard cryopreservation protocols using these approaches

are outlined in **Subheading 3**. Alternative strategies have been developed that may be employed to cryopreserve taxa that are recalcitrant to conventional methods (**9,10**); however, these are not outlined here (for further information on encapsulation/vitrification methods applicable to algae, *see* Chapter 12).

2. Materials

1. Laminar flow cabinet and appropriate facilities for following good microbiological practice.
2. Culture: use late log or early stationary phase cultures if available (*see* **Note 2**).
3. Medium: this is dependent on the nutritional requirements of the alga. For strains currently cryopreserved, the most commonly employed media are detailed in **items 4–6**. In all cases Analar-grade chemicals should be used.
4. BG 11 medium (**Table 1**), used for freshwater cyanobacteria and nonaxenic algae.
5. *Euglena gracilis*: Jaworski's medium (**Table 2**) used for most axenic freshwater algae.
6. Guillard's (f/2) medium (**Table 3**) used for marine algae.
7. Cryoprotectant: 10% (v/v) dimethyl sulfoxide (Sigma-Aldrich) (DMSO) in the appropriate medium. DMSO is cytotoxic and care should be taken when handling. Alternative cryoprotectants are also regularly employed for some strains (*see* **Notes 3 and 4**).
8. Cryovials: 1.8-mL presterilized plastic screw-cap cryovials (*see* **Note 5**).
9. Refrigeration systems:
 - a. Refrigerated methanol bath precooled to -40°C . **Note**: methanol is toxic and flammable.
 - b. Passive freezing unit (Mr. Frosty, Nalge Nunc International, Rochester, NY) and -80°C freezer (*see* **Note 6**).
 - c. A controlled-rate cooler (*see* **Note 7**).
10. Liquid nitrogen Dewar: small 1- to 2-L wide-neck Dewar.
11. Safety equipment: long forceps, cryogloves, cryoapron, and goggles.
12. Storage system: cryogenic storage containers with appropriate storage racks and inventory system suitable for holding cryovials (*see* **Note 8**).
13. Water bath.
14. Fluorescein diacetate (FDA) stain stock solution: 25 mg FDA in 25 mL of methanol (*see* **Note 9**).
15. Microscope equipped with fluorescent lamp and $\times 400$ magnification.
16. Dissecting microscope ($\times 50$ magnification).

3. Methods

All culture manipulations should be carried out following good microbiological practice/aseptic technique in a laminar flow cabinet if possible.

1. Grow cultures in the appropriate medium (*see* **Subheading 2., items 3–6**) under controlled environmental conditions. Flasks (50 mL) containing 30 mL medium should be incubated static at 15°C under a photofluence rate of $25\text{--}100\ \mu\text{mol m}^2/\text{s}$

Table 1
BG 11 Medium

Stock solutions	Per L
NaNO ₃	15.0 g ^a
K ₂ HPO ₄ ·3H ₂ O	4.0 g
MgSO ₄ ·7H ₂ O	7.5 g
CaCl ₂ ·2H ₂ O	3.6 g
Citric acid	0.6 g
Ferric ammonium citrate	0.6 g
EDTA (disodium salt)	0.1 g
Na ₂ CO ₃	2.0 g
Trace metal mixture:	
H ₃ BO ₃	2.86 g
MnCl ₂ ·4H ₂ O	1.81 g
ZnSO ₄ ·7H ₂ O	0.222 g
Na ₂ MoO ₄ ·2H ₂ O	0.39 g
CuSO ₄ ·5H ₂ O	0.079 g
Co(NO ₃) ₂ ·6H ₂ O	0.0494 g

^aMay be omitted for nitrogen-fixing cyanobacteria.

Add 100 mL solution 1, 10 mL each of solutions 2–8, and 1 mL solution 9 to distilled or deionized water to obtain a total volume of 1 L. pH is adjusted to 7.8 prior to sterilization.

(Based on the medium described by Rippka et al. [11].)

(see **Note 10**). Light should be provided on a light:dark cycle; 16:8 h is generally regarded as optimal. Incubate cultures until they reach stationary phase; 30 d is used as a standard culture interval at CCAP.

2. Aseptically transfer sedimented cells with 15 mL medium into a presterilized universal tube. Alternatively, for uniform cell suspensions, centrifuge at 500g for 10 min then decant the supernatant and resuspend the algae in 15 mL fresh sterile medium. Remove a 5-mL aliquot for use as a control for viability assays (see **step 11**).
3. Add 10 mL medium containing 10% (v/v) DMSO in culture medium (bacterial contaminants removed by passing it through a 0.45- μ m filter) to the remaining 10 mL of dense culture and mix thoroughly to give a final concentration of 5% (v/v) DMSO (see **Notes 3, 4, and 11**).
4. Dispense the mixture in 1-mL aliquots into sterile, prelabeled cryovials (see **Note 12**). Seal the vials and incubate at room temperature for 5 min (see **Notes 13 and 14**).
5. First cooling phase (see **Note 15**).
 - a. Passive freezing system method 1: refrigerated alcohol bath. Transfer the filled cryovials to a precooled refrigerated bath (–40°C) and incubate for 15 min (see **Note 16**).

Table 2
***Euglena gracilis*: Jaworski's Medium**

Stock solutions	Per 200 mL
Ca(NO ₃) ₂ ·4H ₂ O	4.0 g
KH ₂ PO ₄	2.48 g
MgSO ₄ ·7H ₂ O	10.0 g
NaHCO ₃	3.18 g
CaCl ₂	2.00 g
EDTA FeNa	0.45 g
EDTA Na ₂	0.45 g
H ₃ BO ₃	0.496 g
MnCl ₂ ·4H ₂ O	0.278 g
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.2 g
Cyanocobalamin (vitamin B ₁₂)	0.008 g
Thiamine HCl (vitamin B ₁)	0.008 g
Biotin	0.008 g
NaNO ₃	16.0 g
Na ₂ HPO ₄ ·12H ₂ O	7.2 g

Add 0.5 mL solutions 1–10 to distilled or deionized water to obtain a total volume of 1 L medium. Then add 0.5 g sodium acetate (trihydrate), 0.5 g Lab-Lemco powder (Oxoid L29), 1.0 g Tryptone, and 1.0 g yeast extract. (Based on the medium described by Thompson et al. [12].)

Table 3
Guillard's (f/2) Medium

Stock solutions	Per L
NaNO ₃	7.5 g
NaH ₂ PO ₄ ·2H ₂ O	5.65 g
Na ₂ EDTA	4.36 g
FeCl ₃ ·6H ₂ O	3.15 g
CuSO ₄ ·5H ₂ O	0.01 g
ZnSO ₄ ·7H ₂ O	0.022 g
CoCl ₂ ·6H ₂ O	0.01 g
MnCl ₂ ·4H ₂ O	0.18 g
Na ₂ MoO ₄ ·2H ₂ O	0.006 g
Cyanocobalamin (vitamin B ₁₂)	0.5 mg
Thiamine HCl (vitamin B ₁)	100.0 mg
Biotin	0.5 mg
Na ₂ SiO ₃ ·9H ₂ O	40.18 g

Add 10 mL solution 1 and 1 mL each of solutions 2–4 to filtered natural seawater obtained from a clean offshore/nonestuarine source, to obtain a total volume of 1 L. Adjust pH to 8.0 prior to sterilization. Add 1 mL of solution 5 to the medium prior to pH adjustment and sterilization, if the medium is to be used for diatoms. (Based on the medium described by Thompson et al. [12].)

- b. Passive freezing system method 2: “Mr. Frosty” (*see Note 6*). Place isopropanol, as per instructions, in the reservoir adjacent to the chamber containing cryogenic vials of a “Mr. Frosty” and place in a refrigerator overnight to equilibrate at approx 4°C. Quickly transfer the cryovials containing cryoprotectant-treated algae into the “Mr. Frosty” and immediately transfer to a –80°C freezer. Leave the container undisturbed for 1.5 h, by which time the temperature of the contents of the vial is less than –50°C (*see Note 17*).
 - c. Controlled-rate cooling. A simple protocol calls for cooling the chamber at 1°C/min from 20 to –40°C, then dwelling at –40°C for 30 min prior to transferring cryogenic vials to liquid nitrogen (*see Notes 18 and 19*).
6. Second cooling phase. On completion of the first cooling phase as outlined above (*see step 5a–c*). Transfer the vials rapidly, using forceps, to a wide-necked Dewar containing liquid nitrogen. Then, transport the vials in the Dewar to the storage system.
 7. Transfer the vials rapidly, using long forceps, to the storage/racking system. Storage is generally in the liquid phase of liquid nitrogen (*see Note 20*).
 8. A full inventory/stocklist including the locations of the vials within the storage system should be maintained. This can most easily be retained either on a computer database as card indices or on any other appropriate systems.
 9. Thawing. Transfer stored cryovials (as in **step 6**) to a Dewar containing liquid nitrogen for transport and temporary storage. When ready, place the vials in a pre-heated water bath (40°C) and agitate until the last ice crystal has begun melting (*see Note 21*). On completion of the thawing remove immediately and transfer to the laminar flow cabinet (*see Note 22*).
 10. Wipe the cryovial with 70% (v/v) ethanol to sterilize the outer surface of the vial. Aseptically transfer the contents into 30 mL of appropriate sterile medium and incubate as detailed in Method section 1 (*see Notes 23 and 24*).
 11. Viability assays.
 - a. Vital staining (*see Note 25*). It is necessary to dilute out cryoprotectant by transferring the thawed culture into 9 mL of appropriate sterile medium and incubating for up to 24 h prior to staining (*see Note 25*). Add 50 µL of FDA stain stock solution to 1 mL culture, incubate at room temperature for 5 min, and observe by blue-light fluorescence microscopy (*see Note 26*). Viable cells fluoresce green (FDA positive) and nonviable cells appear red or colorless. Viability is expressed as a percentage of control (nontreated unfrozen culture; **step 2**) vs FDA-positive cells.
 - b. Colony formation in agar (*see Note 27*). Dilute control cultures, i.e., the untreated unfrozen starting culture (**step 2**) 1:1 with sterile medium. Transfer 1-mL aliquots of logarithmic dilutions of control and freeze/thawed cultures (*see Note 28*) into sterile Petri dishes (50-mm diameter). Add approx 2.5–4.0 mL of 1.0% (w/v) agar in the appropriate medium at 40°C; agitate gently to mix culture and agar (*see Note 29*). When the agar has gelled, seal the Petri dishes with Parafilm™ and incubate under standard growth conditions as detailed in **step 1** (*see Note 30*). Colonies are counted using a dissecting microscope (magnification ×50) and the viability of thawed cultures expressed as a percentage of control culture.

4. Notes

1. At low cell densities photoinhibition may occur as a result of the lack of self-shading by other algal cells. This, in turn, may result in the death of cells that have survived cryopreservation.

Where cultures are not axenic, low post-thaw viabilities may result in the overgrowth of the alga by the competing associated microbial flora.

2. Stationary-phase cultures are denser, thus reducing the time required to reestablish a viable culture. Also, stationary-phase cultures may contain intracellular storage products, including lipids, which may act as additional cryoprotectants.
3. DMSO may also be used at 10% (v/v) final concentration. Alternatively, glycerol at either 5 or 10% (v/v) or methanol at 5 or 10% (v/v) may be used. Glycerol is normally sterilized by autoclaving at double the final concentration in culture medium. DMSO and methanol are sterilized by filter sterilizing through an alcohol-stable 0.2- μ m filter.
4. Choice of cryoprotectant is largely dependent on its effectiveness and its cytotoxicity to the algal strain being frozen. Concentrations of methanol or DMSO less than 2% (v/v) are seldom effective as cryoprotectants, whereas concentrations higher than 12% (v/v) are often toxic. Within this range the most effective concentration varies greatly among species, sometimes even among closely related strains.
5. Costar vials (Cambridge, MA) have an internal rubber O-ring that appears to reduce the likelihood of liquid nitrogen leaking into the vial. Nitrogen leakage may result in contamination of algal cultures when stored under liquid nitrogen. Leakage can also lead to the possible rupturing of the vial on thawing, which has obvious safety implications (*see Note 22*). It is important not to overtighten or undertighten the caps, as this will result in leakage. An additional step that may be employed to minimize the risk of leakage is to use Cryoflex (Nunc). This will form a seal and prevent leakage into the vial.
6. Commercially available controlled-cooling canisters, such as “Mr. Frosty” and “Handi-Freeze” (Taylor Wharton, Nottingham, UK), are inexpensive and when used correctly have highly reproducible cooling rates. However, care should be taken on transferring them to/from the freezer and placing them in the freezer to avoid any loss of isopropanol, as this will alter the cooling rates of the unit.
7. A variety of commercial instruments (e.g., Biotronics, Leominster, UK; Planer Products, Sunbury, UK; Cryomed, USA; Gordinier Electronics, Roseville, MI; CryoLogic, Musgrave, Victoria, Australia) allow accurate control and manipulation of the cooling regime.
8. Vials must be stored at less than -135°C . This is achieved by storage either in the liquid or vapor phase in an insulated Dewar, or alternatively, in a more sophisticated autofill crystore. At CCAP, liquid-phase storage is preferred as this ensures that stored material is always maintained at below -135°C .
9. Prepare a standard FDA stock solution (0.001% [w/v]) by first dissolving 25 mg FDA crystals in a few drops of acetone and making up to the final volume (25 mL) with methanol.

10. Cyanobacteria and red algae, particularly when physiologically stressed, should be cultured at relatively low light levels. Light intensity is less critical for most other eukaryotic algal groups.
11. A few small, unicellular Chlorophyceae, such as *Chlorella protothecoides* Krüger, can withstand direct immersion into liquid nitrogen without a cryoprotectant or any control over the rate of cooling (8). Some strains of *Chlorella* can be preserved by incubating a culture in 5% (v/v) DMSO at ambient temperature for 5 min in a cryogenic vial, and then plunging the vial directly into liquid nitrogen.
12. Cryogenic vials are best labeled using a fine-tip, alcohol-resistant permanent marker. Despite limited space, the algal name or identifying number and the date of freezing should be labeled on each frozen vial. If multiple batches of vials are frozen, a batch number should also be written on each cryogenic vial and a permanent record should be kept of each batch.
13. For some slow and nonpenetrating cryoprotectants, e.g., glycerol, cultures are incubated for 30 min in the presence of cryoprotectant prior to cooling. For some sensitive strains it has been suggested that cryoprotectants should be added at 0°C by incubating on ice (10).
14. Many algae can be cryopreserved directly on an agar slant. A small volume (0.3–0.5 mL) of sterile agar medium is aseptically transferred to a sterile 2-mL cryogenic vial and allowed to solidify as a slant. An algal culture is spread or streaked on the surface of the solidified agar and incubated under normal growth conditions. The culture is ready for cryopreservation after it has grown into a heavy streak or a lawn, and many cyanobacteria and unicellular chlorophytes can be cryopreserved after 2–3 wk. In preparation for cryopreservation, growth medium containing an appropriate cryoprotectant solution is added slowly to the cryogenic vial in order to minimize the disturbance of algae growing on the agar surface. This preparation of algae can then be cryopreserved like liquid cultures. To revive the culture, rapidly thaw the vial, gently decant the liquid, and add fresh culture medium to the vial. If the alga adheres to the agar surface, then the liquid medium can again be decanted and the cryogenic vial incubated under normal culture conditions. Viable algae remaining on the agar surface typically grow into a lawn within 2–3 wk (13).
15. For most protocols a Step 1 terminal temperature of –40°C is sufficient. However, when methanol is used as the cryoprotectant at the UTEX algal collection at –45 to –55°C is used as a terminal temperature (13).
16. For some algae, including members of the Prasinophyceae, incubation for 30 min at –40°C is used.
17. The chamber cools at approx –1°C/min over a temperature range of 0°C to –50°C. For some strains it may be desirable to leave the container in the –80°C freezer for only 60 min, at which time the content of the cryogenic vial reaches approx –40°C. For some strains it has been found to be advantageous to leave the Mr. Frosty for up to 4 h at –80°C. A convenient protocol suitable for many strains of microalgae is to remove the canister after the desired temperature is reached and quickly transfer the frozen vials to an ultra-cold storage vessel.

18. One can use cooling rates of up to $-10^{\circ}\text{C}/\text{min}$ for many members of the Chlorococcales. Slow cooling rates ($0.5\text{--}5^{\circ}\text{C}/\text{min}$) are generally used for many larger and more complex microalgae (14–16).
19. A more complex cooling program successfully employed at CCMP for marine strains involves cooling the contents of cryogenic vials from ambient temperature to 4°C at $-1^{\circ}\text{C}/\text{min}$, then holding the temperature constant for up to 5 min. This dwell time is especially effective for cold polar strains and is sometimes required for adequate penetration of the cryoprotectant. Vial contents are next cooled at $-1^{\circ}\text{C}/\text{min}$ until they reach -9°C . Seawater remains as super-cooled liquid at that temperature. The cooling chamber is then cooled rapidly to -45°C in order to quickly drive the contents of cryogenic vials down to -12°C . This induces ice nucleation and rapidly removes the latent heat of fusion. The contents of vials are then cooled at $-1^{\circ}\text{C}/\text{min}$ until they reach -45°C , which is below the eutectic point (see Glossary). The vials are then cooled rapidly to -90°C and finally transferred from the cooling chamber to a liquid nitrogen storage system (8).
20. Cryopreserved cultures in cryogenic vials are generally maintained for long periods of time in one of three ways: (1) submerged in liquid nitrogen (-196°C), (2) in the cold vapor phase above liquid nitrogen (approx -165°C), or (3) in an ultra-cold electrically driven freezer (-150°C).
21. Using a floating vial holder (e.g., Nalgene 5974-4015) reduces the possibility of contamination caused by contact with the water in the waterbath.
22. Liquid nitrogen leakage into the vial is potentially dangerous. On thawing, the nitrogen will evaporate and this could cause the vial to explode. Care should be taken when handling vials containing nitrogen and full-safety equipment (gloves, apron, goggles, and so on) should be used.

Liquid nitrogen may contain low levels of viable bacteria and these, in turn, may contaminate axenic cultures if leakage occurs. Where alternative frozen specimens are available, vials containing liquid nitrogen should be discarded.
23. For cultures that do not settle rapidly, the cryogenic vial may be subjected to gentle centrifugation in order to pellet the culture. Discard the supernatant and dilute the pellet with fresh culture medium.
24. Allow the recovered culture to remain in darkness, or in subdued light (normal room light is generally acceptable, but not in close proximity to a source of artificial illumination, or a window exposed to bright outdoor light) for several hours, preferably overnight. Then place the culture under normal culture conditions and expect growth of viable cells to resume within 1–2 d. The addition of a small amount of yeast extract (0.1 g/L), proteose peptone (0.1 g/L), or soil extract (10 mL/L) sometimes enhances viability of axenic cultures.
25. An incubation period that allows repair of sublethal damage, but which is too short for cell division to occur, will give a more accurate index of viability when using vital staining rather than staining immediately after thawing.
26. Temperature levels of the specimen increase rapidly during fluorescence microscopy and cells will die within 1–2 min. Observations and counts must be performed rapidly to prevent an underestimate of the viability level.

27. For many algae, particularly nonmotile algae that do not survive embedded in agar, spread plates of logarithmic dilutions may be used instead of pour plates. Spread 0.1 mL logarithmic dilutions of control and frozen and thawed culture onto the surface of Petri dishes (50 mm) containing an appropriate agar (1.5% [w/v]) solidified medium. Incubate and enumerate as detailed for pour plates.
28. A sufficient number of algal units should be transferred to the 50-mm plates to yield between 50 and 200 colonies per plate, because that provides statistically meaningful numbers, yet allows convenient and accurate counting.
29. Exposure time to the hot agar should be minimized; 40°C for more than a few minutes will be lethal for many nonthermophilic algae. For temperature-sensitive strains, melted agarose with a lower melting point may be mixed with the culture prior to transferring to plates.
30. For most algae, incubation at 25°C will reduce the period required to obtain discrete countable colonies.

Acknowledgment

The author gratefully acknowledges the support of the EU for the COBRA project 0LRI-CT-2001-01646.

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