Cryopreservation of microalgae & cyanobacteria employing a controlled rate cooler

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The traditional approach to cryopreservation involves exposure of cells to sub-zero temperatures using gradual (slow), and controlled rates of temperature reduction in the presence of a cell penetrating cryoprotective agent (CPA). The success of the method is dependent upon minimising the effects of two potentially injurious factors:

(1) Ice (intra- & extra-cellular)

(2) Dehydration

Cells are stored at ultra-low temperatures, below the point at which ice crystals can form, or grow.

PREAMBLE. For all cryopreservation procedures it is optimal to employ a healthy, vigorous, relatively dense, preferably late log-phase, or early stationary-phase culture. Where practicable axenic strains, or those with low levels of bacterial contaminants, should be employed and those contaminated with other eukaryotes avoided.

1. Prepare the CPA/ cryoprotectant solution. Most commonly either 10-ml of 10% (v/v) dimethyl sulphoxide (DMSO), or 10-ml of 10% (v/v) methanol, is added and slowly mixed into the appropriate sterile culture medium and filter-sterilised into a sterile glass universal vial.

   As the cryoprotectant is subsequently diluted 1:1 with the culture (see 2.) a final cryoprotectant concentration of 5% (v/v) is employed. A range of alternative CPAs have been successfully employed for different biological materials and if trials are undertaken empirical experimentation is required to optimise the concentrations to be used and duration of incubation. For some marine microalgae a final concentration of 5-10% (v/v) glycerol may be used as cryoprotectant. In these cases a CPA of up to 20% (v/v) needs to be sterilised, as this is extremely viscous filter-sterilisation is impracticable and pre-prepared cryoprotectant in the appropriate culture medium should be sterilised by autoclaving at 121^{o}C for 15 min.

2. Aseptically transfer 10-ml of dense culture into a universal vial and add 10-ml of sterilised cryoprotectant solution. Seal the vial and invert twice to ensure thorough mixing.

3. Aseptically decant 1-ml aliquots into cryovials and incubate for 10 min at room temperature.

   At SAMS 15 vials per algal strain are normally filled and processed (see below).

John G. Day
Scottish Association for Marine Science (SAMS), Oban, UK

Additional Notes

This method has been successfully employed for a wide variety of algae including: cyanobacteria, unicellular & filamentous green algae, Prasinophytes, brown algae, Euglenophytes and diatoms.

Apparatus: incubators with controlled light/dark cycle; a class I biological safety cabinet; a controlled rate cooler; a small (1-l) dewar containing liquid nitrogen; safety clothing; a heated water-bath; storage cryostat/refrigerator; long forceps.

Culture medium: medium appropriate for the taxon and its osmotic origin.

Plasticware/glassware: membrane filters (0.5-µm pore size); sterile syringes; disposable pipettes; 2-ml cryogenic tubes; glass universal vials (20-ml).

All chemicals should be Analytical grade.

Additional information:

***Day JG** (2014) Cryopreservation of cyanobacteria. In: Cyanobacteria: An economic perspective. Sharma NK, Rai AK & Stal LJ (eds) WILEY Publications. pp 319-328.*

***Day JG** (2007) Cryopreservation of Microalgae and Cyanobacteria. In: Day JG & Stacey GN (eds) Cryopreservation and Freeze-drying Protocols. Humana Press. pp 139-149.*

***Day JG and Brand JJ** (2005) Cryopreservation Methods for Maintaining Cultures. In: Algal Culturing Techniques. Andersen RA (ed) Academic Press, New York. pp 165-187.*

4. Programme the controlled cooler: Start temperature 20°C; ramp, cool at -1°C min^{-1} to -40°C; dwell, hold at -40°C for 10 min.

   For some taxa optimal cooling rates need to be empirically ascertained. In general slower cooling rates (<-1°C min^{-1}) are optimal for larger cells (>25µm diameter). In general mechanical seeding of ice is not used for algal cryopreservation, but if this is an option in the system employed, empirical experimentation may be used to ascertain if it results in higher post-thaw viability. However, it should be noted that mechanical seeding is routinely employed for the conservation of a wide range of biological resources and ensures greater reproducibility of the protocol. This in turn should reduce batch to batch variation and potentially variation in viability levels within a single batch.

5. Start the programme to purge the cooling chamber with nitrogen vapour and to allow the system to stabilise at the start temperature.

   For most modern controlled coolers this is now pre-programmed prior to initiating a cooling profile.

6. On reaching the start temperature, (most systems have an audible alarm) transfer the cryovials to the cooling chamber of the programmable cooler and initiate the cooling ramp.

   For the majority of published protocols the start temperature is taken as nominal room temperature, most commonly 20°C. However, for some larger taxa such as Euglena, *where very slow cooling rates are employed and methanol is used as the CPA of choice, prolonged exposure to the cryoprotectant can be damaging. Therefore initial cryoprotection is performed on ice and the cooling protocol initiated at 0°C.*

7. After the end of the programme an alarm will sound. Rapidly transfer the cryovials to a small dewar containing liquid nitrogen using long forceps.

   It is essential that the cryovials are not allowed to warm up prior to plunging into liquid nitrogen.

8. Samples for storage should be transferred to the cryostat (ultra-cold freezer) in the liquid nitrogen containing dewar. The transfer of cryovials to the storage system should be performed rapidly using long forceps.

   Storage temperature is critical and should normally be <-130°C, samples should never be allowed to warm to above -90°C, as this will significantly reduce the viability of stored cells. It is advisable that replicate samples are stored in at least two separate "refrigerators". At SAMS 10 cryovials are normally stored in a Working bank, two vials are stored in a Master, or back-up, bank and three are used to check viability/ efficacy of the protocol.

9. To recover cultures, the cryovials are thawed by placing in a pre-heated water-bath (40°C) and agitating until the last ice crystal has just melted.

   For most marine taxa it is important not to prolong their incubation at 40°C. Alternative, slower warming e.g. in a 25°C water-bath may be beneficial for some strains, but in general rapid warming is optimal as it avoids/ minimises ice crystal regrowth.

10. On thawing rapidly transfer to a clean laminar flow/ biological safety cabinet and wipe the outside of the vial with 70% (v/v) ethanol.

    Note: there may be high levels of viable bacterial and fungal spores in liquid nitrogen that may contaminate recovered cultures.

11. Using a disposable plastic pipette transfer the 1-ml of thawed culture into a suitable, labelled, culture vessel containing 10 - 20-ml of an appropriate sterile medium. Cover in aluminium foil and re-label with strain designation and date.

    This dilution minimises any potentially deleterious effects that sensitive taxa may experience due to longer-term exposure to the cryoprotectant solution. To prevent further biochemical-based injuries it is optimal to incubate for a period in the dark, for each organism this may be required to be optimised empirically, with optimal dark phases ranging from 12 – 168 h.

12. Incubate at standard culturing temperature for the cryopreserved organism. After the dark phase partially remove the foil and after a further 24 - 96 hours remove all the foil covering.

    It is important to ensure that cells are not subjected to light levels likely to induce photo-oxidative stress during the recovery phase. It is worth noting that cell numbers are generally very low and, particularly for strains with poor recovery levels, that there is little "self-shading". For some larger organisms a washing, or medium refreshing, stage is helpful to reduce nutrient levels/bacterial growth induced by the lysis of cells killed by the cryopreservation process. This can prevent overgrowth by commensal bacteria.

13. After an appropriate period (2 - 8 weeks, depending on the strain) a normal culture should be obtained. This may be maintained by routine serial transfer, or employed for experimental use.

    It is important to check the recovered culture for any contaminants and to ensure normal phenotypic and physiological features (motility, cell structures etc.).

EnAlgae Output WP1A4.03

How to cite this article: Day, J.G. (2015) Cryopreservation of microalgae & cyanobacteria employing a controlled rate cooler. EnAlgae output WP1A4.03, 3pp. http://www.enalgae.eu/public-deliverables.htm.

This document is an output from the Energetic Algae ('EnAlgae') project, which has received European Regional Development Funding through the INTERREG IVB NWE programme.