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Cryopreservation of Microalgae

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[1]Realizing Increased Photosynthetic Efficiency (RIPE)

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ABSTRACT

Cryopreservation protocol for the microalgae Ostreococcus tauri for long term storage.
Cell viability of O. tauri at -196 for x months = unknown
Regeneration growth rate of O. tauri at -196 for x months = unknown

Additional Information

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GUIDELINES

Not all species of algae can be successfully cryopreserved. Cryopreservation protocols, cryoprotectant agents, viability on thawing, and growth rate on thawing will differ for each species. This protocol has not been widely tested. Typical cell recovery for microalgae that have been tested for cryopreservation viability is 20-80%.

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 Phytoplankto nin the U.S.A, ccmp.bigelow.org, 2009). (Youn, J. & Hur, S., 2009)

Overview of Cryopreservation

MATERIALS TEXT

- Laminar Flow Hood, NuAire Class II, Type A2
- -80 C Capable Freezer, Ultra Low Temperature Freezer Thermo Scientific Revco UxF ,Build 30.04, Unit P302957
- Mr. Frosty freezing container (Fisher Scientific 15-350-50)
- Liquid nitrogen tank (American Biotech Supply 33L ABS-ET 33)
- **Racks for LN2 tank**
- **Sample storage boxes for LN2 tank**
- Water bath (Grant Instruments Model JBN12 US)
- Optional: Shop vac, helps remove vapor from LN2 tank to more quickly find samples. (Stinger Model#HD2025)

Chemicals and Solutions

- Culture Medium, specific to organism
  *O. tauri: Prepared ASW + K Medium (https://www.protocols.io/view/keller-k-medium-in-artificial-sea-water-for-cultur-brv7m69n)
- Dimethylsulfoxyde (DMSO), CAS 67-68-5
- Methanol, CAS 67-56-1
- Liquid nitrogen, CAS 7727-37-9

Supplies

- 2 mL Cryogenic tubes (Fisher-Scientific 10-500-26)
- Labels, liquid nitrogen resistant (Fisher-Scientific 15-910-A)
- Aluminium foil (Fisher Scientific 01-213-100)
- 22 um Syringe Filter, MCE Membrane, 23 mm diameter, Sterile Millex, MilliporeSigma (Fisher Scientific SLGSR33SB)
- 60 mL sterile single-use syringe, Fisherbrand (Fisher Scientific 14-955-461)

SAFETY WARNINGS

- Liquid Nitrogen safety hazards include extreme cold, asphyxiation, and explosion; resulting in sample loss and personal injury. Review cryogen safety and biological sample storage in liquid nitrogen safety documents, and complete the Compressed Gases and Cryogens Safety Training module. Wear appropriate PPE when handling including safety goggles, lab coat, thermal gloves, and closed toe shoes.

- Dimethyl sulfoxide is rapidly absorbed through skin. Read safety data sheet before handling. Wear appropriate PPE when handling including nitrile gloves and lab coat.

BEFORE STARTING

Establish and maintain healthy populations of algal culture.

Cryopreservation

1 Verify culture concentration daily. Cryopreservation should be made towards the end of the log phase or 1-2 days after the beginning of the stationary phase.

2 ⊛

Optional: Induce cold adaptation by placing algal cultures at **4 °C** for 3 days.

Freezing resistance of some algae has been shown to increase with a decrease in ambient culture temperature, this phenomenon being analogous to the cold-hardening of higher plants during the cooler autumn months (Taylor et al.) ((Mortain-Bortrandet al.)

3 In laminar flow hood, dilute algal cell concentration to 1×10^{6} cell mL^{-1} in **1 mL** of filter sterilized medium prepared with selected cryoprotectant and additives.

It is theorized that the diminished viability of microalgal cells cryopreserved at high cell densities is caused by the enzymatic release of a cell-wall component. Of the tested cell densities in several species, the highest recovery was seen at 1×10^6 cell mL−1. There is the possibility that further dilution could further improve recovery rates. Although improved viability at lower cell concentrations appears advantageous, lower cell concentrations mean a substantial reduction in the absolute numbers of colonies, even with an improved survival, and so is of limited interest. (Bui et al. 2013) (Piasaki et al. 2009)

4 In a laminar flow hood, add filter sterilized [M]**7 % (v/v)** DMSO (Diméthylsulfoxyde) to a final concentration to **1 mL** of culture and transfer to a 2mL cryogenic tube.

⚠ DMSO abosrbs rapidly through skin. Wear appropriate PPE including nitrile gloves, lab coat, and safety eyewear.

The best cryoprotectant varies by species, strain, storage condition, and storage length. The three most common cryoprotectants for microalgae are DMSO, methanol, and glycerol.

Typical ranges found that <2% of a cryoprotectant was ineffective and >12% was typically toxic. (Abreu et al.) 5% and 10% are the most commonly used concentrations for methanol and DMSO. Rate of DMSO was found to be

optimized between 6-7% for multiple strains. (Bui et al. 2013)

Methanol may induce light sensitivity. (Alexandra et al. 2009)

5

Add filter sterilized cryoprotectant additives to cultures, if using.

In some species, using a combination of cryoprotectants or cryoprotectant additives, such as sugars or antioxidants, has shown positive results for thawed cell viability. The positive additive affect is not well studied and is highly variable by species, strain, and protocol.

6 Allow algae sample with cryoprotectant to equilibrate for **00:05:00** at room temperature (**Room temperature**) 5m

Both DMSO and MeOH permeability of algal membranes and cell absorption is higher at warmer temperatures. (Tanka et al. 2001)

7

Progressively freeze the algae at a target rate of -1^{o}C/min starting at **20 °C** by placing the samples in an isopropanol filled, pre-chilled (**4 °C**) Mr. Frosty freezing container and moving to a **-80 °C** freezer for 2-4 hours.

Do not move the freezing container during progressive freezing. Sloshing or spilling of isopropanol will cause an inconsistent cooling rate.

Ideal end point for progressive freezing varies across the literature from -30C to -55C and occurs in freezers ranging from -40 to -80C. Results in these ranges seemed comparable. -80C was selected based on our available equipment.

2-3 hour passive cooling led to higher cell survival than 1hr cooling or overnight cooling in C.Merolae. (Ohnumaet al.)

Optimal freezing rate may be closer to 0.5^{o}C/min but this rate is only achievable using a programmable progressive freezer. (Fleck et al. 2003) In general slower cooling rates (<-1°C min.-1) are optimal for larger cells (>25µm diameter). (Day, SAMS)

Best practice is to use a programmable progressive freezer. Cost of a base model progressive freezer is $26000. A Mr. Frosty freezing container is about $100.

8

When the cycle is finished, immediately plunge the cryogenic tubes into liquid nitrogen. **It is essential that the cryovials are not allowed to warm up prior to plunging into liquid nitrogen.**

Cryogens and liquid nitrogen pose a safety hazard. Wear appropriate thermal gloves, safety goggles, lab coat, and close toed shoes.

Evaporate from liquid nitrogen can pose an asphyxiation risk. Never transfer or use liquid nitrogen in an enclosed space.

9 Place cryogenic tubes in cryoboxes and store in a **-150 °C** freezer and/or in a liquid nitrogen container for long-term storage.

Storage temperature is critical and should be < -137 °C .

It is advisable that replicate samples are stored in at least two separate cold storage locations. Samples can be stored at **-150 °C** in a mechanical freezer, at **-165 °C** in the vapor phase of a liquid nitrogen dewar/cabinet, or at **-195 °C** submerged directly into liquid nitrogen. All three methods have advantages and disadvantages. Best practice would be to store replicate samples in two different cold storage locations and using two different types of cold storage.

Culture Regeneration 1m

10 Allow vials to warm at **Room temperature** suspended in the air for **exactly** **00:01:00** then plunge tube in a 1m **40 °C** water bath just until melted.

For most marine taxa it is important not to prolong their incubation at 40^{o} C. Alternative, slower warming in a 25^{o} C water-bath may be beneficial for some strains, but in general rapid warming is optimal as it avoids/ minimises ice crystal regrowth. (Day, SAMS)

A one minute warming in the air before immersion in the 40^{o}C water bath improved viability by 60% over immediate immersion for diatoms. A preliminary slow thaw is likely to have permitted slow glass relaxation avoiding potentially lethal fractures and recrystallisation. The second stage of rapid warming to ambient temperature, avoids ice crystal growth that could cause further damage to the algal cells. (Fleck 2013)

Roscoff recommends thawing at 25-30 C.

11 As soon as ice in the tube has melted, quickly and thoroughly clean the outside of the tube with **[M]70 % (v/v)** ethanol and in a laminar fume hood aseptically transfer the cells into a prepared flask containing at least **20 mL** of fresh sterilized culture medium.

12 Surround the flasks with aluminum foil to keep them in the dark and put them at the optimal temperature for the culture.

13 After 24 hours, remove the aluminum foil. Grow them in a reduced light intensity (8 µE m−2 s−1) for two weeks. Alternatively SAMS places cultures in complete dark for 24 hours, then allows half light for 96 hours (partially removes foil), before returing to full light (Day, SAMS).

During the recovery phase lower light intensities (~8 µE $m^{-2}s^{-1}$) for two weeks were found to be optimal for growth. The light is beneficial for recovery even in the presence of a heterotrophic carbon source, but above ~8 µE $m^{-2}s^{-1}$the cells in the recovery phase are very sensitive to photodamage. It is hypothesized there is a compromise between the beneficial effect of light (particularly for ATP production) on cell viability and the harmful effect of light-mediated damage on photosynthetic membranes where integrity and repair mechanisms may be compromised by freeze-thaw damage. (Bui et al. 2013)

Both Roscoff and SAMS recommend only 24 hours in the dark but for benthic diatoms recovery under lower light levels (3–8µmol photons $m^{-2}s^{-1}$) resulted in increased levels of post-thaw viability (4.5±2.1%). The highest post-thaw viability levels were observed for samples that had been incubated in complete darkness for 4–7days (59±8.3%). It is hypothesized that recovery was significantly enhanced by removing the potentially injurious effects of uncoupled photosynthetic activity and photooxidative stress. (Buhmann et al.)

14 Observe the cultures every other day (by colour and/or by flow cytometry and/or by microscopy).

15 Culture recovery can take 2-8 weeks for normal culture that was healthy at time of cryopreservation (Day, SAMS).