Progress Report on Cryopreservation at the University of Toronto Culture Collection of Algae and Cyanobacteria

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SUMMARY: The University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC) has recently begun cryopreservation of its valuable strains. A Percent-Growth Protocol has been developed with which 15 strains of algae have been successfully frozen and revived. Two other strains including a freshwater diatom, *Nitzschia palea*, are currently in the process of being tested. Eventually, we would like to have most of the strains that are available at UTCC frozen in order to maintain their valuable genetic traits and to reduce the amount of space and work it takes to maintain cultures.

Introduction

The University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC) is Canada's national service collection of freshwater algae and cyanobacteria. It currently maintains about 500 strains by serial transfer in liquid culture and on agar plates or slants. In order to reduce the amount of work it takes to maintain these cultures and ensure their long-term viability, we have recently begun to use cryopreservation as a means of preserving them. Cryopreservation or the "long-term preservation of cellular viability at low temperature" (Morris, 1981) is the most practical and most widely used method of preserving microscopic algae. It has numerous advantages including economies of time, space, materials, and most importantly, avoidance of any genetic drift (Morris, 1981), which can occur in liquid and agar cultures over long-term maintenance by serial transfer. Also, as long as the culture is not directly in contact with the liquid nitrogen, possibility of contamination during storage is minimal.

This paper is a report on the progress of cryopreservation practices at UTCC. The objective of this cryopreservation project is to freeze as many of our strains as possible and to also achieve good recovery rates. Since one of the main functions of UTCC is to provide algal cultures for distribution to researchers in universities, government, and industry, it is important to know how long it would take after thawing cultures to have them grow to a suitable density for shipping. We are investigating not only whether strains can survive cryopreservation but also how long it takes after thawing to grow them to 5 times their original concentration at the time of freezing.

Methods and Materials

Since "algae cells from actively dividing exponential phase are more sensitive to freezing injury than are cells from older stationary phase" (Morris, 1981), we used cultures that had passed the exponential growth phase. Strains were grown in 125 ml flasks on a shaker at 23°C under fluorescent lamps (GE Cool-White) at about 100 microEin/m^2/sec^{-1} for approximately one month or until the concentration reached at least $1x10^6$ cells/ml before freezing. If the concentration of cells was too high, the culture was diluted to $1x10^6$ cells/ml with culture media. Strains that were colonial or excessively clumped were also frozen after approximately one month of growth but without counting the cell concentration.

Morris (1981), Day & McLellan (1995), Day et al. (2000), as well as others have found that the most successfully cryopreserved strains of algae were frozen using two-step protocols involving cooling to an intermediate subzero holding temperature (commonly –30 or –60°C) prior to plunging into liquid nitrogen. Thus, a simple two-step protocol was employed in freezing our strains[*]. With the aid of methanol (MeOH) as a cryoprotectant, usually at 5% (v/v with medium) concentration, the cultures were cooled 1°C/min using NALGENE Cryo 1°C Freezing Containers (Mr. Frosty) for 2 hours to approximately –60°C (at –86°C in a Sanyo Freezer). Culture media containing various concentrations of reagent grade MeOH were filter-sterilized through an Acrodisc (PALL Acrodisc PF, 32 mm Syringe Filter with 0.8/0.2 μm Supor Membrane) prior to use. To each of the cryovials (2-ml Simport with external threads) 0.75 ml of culture medium containing 10% MeOH was added. The control cryovials received 0.75 ml of culture medium. Controls were prepared only when the percent-viability or percent-growth of a culture was being tested in order to see whether the cryoprotectant was necessary for good recovery. When it was known how long it took for the culture to grow to 500%, cultures were frozen for future use without any controls. Both MeOH and culture media were refrigerated before use. In preparation for freezing, 0.75 ml of culture was added to each of the cryovials. This was done very quickly as the cells may be damaged by the cryoprotectant when not frozen. Before use, Mr. Frosty containers were cooled in the –86°C freezer for approximately 1 hour. All the cryovials were placed into a Mr. Frosty that had been pre-filled with 250 ml of 99-100% isopropyl alcohol (IPA) and then put into the –86°C freezer for 2 h. (Note that if 70% IPA is used, it will freeze at –86°C, thus not cooling the cultures at the appropriate rate.) All the cryovials were placed into the corresponding Cryobox (as specified by the Cryoware Inventory System[*]) in the vapour phase of liquid nitrogen in a CBS Cryosystems 4000 Series Cryotank and kept frozen until needed.

To test for percent-growth, 4 vials were thawed after 1 week and another 4 were thawed at 1 month after freezing. All of these procedures were done in very dim light as cells may be damaged by exposure to bright light when in cryoprotective solution. The cryovials were removed from the cryotank and were held in a Tek-Pro water bath at 37°C for 5 min or until fully thawed. The cryovials were then centrifuged in an IEC Clinical Centrifuge for 5-7 min at speed 3 (~3000 rpm). If no pellet was visible at the bottom, the vials were centrifuged for up to 10 min. To wash out the cryoprotectant 1 ml of the cryovial contents was removed under sterile conditions in a laminar flow hood and replaced with fresh sterile culture medium; care was taken not to disturb the pellet at the bottom. This was repeated twice. After gentle agitation, 1 ml of culture was taken out and dispensed into 4 ml of fresh sterile culture medium (this medium had been refrigerated in screw-cap glass tubes that were sealed with Parafilm when not in use). The cultures were then kept at 23°C in the dark for 2 days after which they were placed in continuous fluorescent light to grow at room temperature.

After each thawing procedure, a weekly count for each of 4 weeks was done on each culture taking care not to contaminate it. From these cell counts, the percent-growth of each culture was calculated. To test for sterility, each culture of axenic strains (not

[*] Please contact the curator of UTCC for the detailed Percent-Growth Protocol for Algae (last modified July 31, 2004).

[*] see Appendix II

including the controls) was plated on a sterility test agar plate (growth medium with addition of 1g/L glucose, 1g/L peptone, and 0.3g/L yeast extract) after 4 weeks of growth. These plates were monitored for at least 2 weeks to check for contaminants.

In order to keep track of the frozen cultures, Cryoware Inventory System (Appendix II) and Cryo Data Sheets (Appendix III) were filled out every time that a strain was frozen. The Cryotank Monthly Inventory was done each month (Appendix I).

Results

Initially, several strains of green algae were tested for percent-viability, the number of viable cells per ml post-thaw versus the number of cells per ml in the cryovial before thawing. The following strains were tested in this way: 121, 123, 120, 89, 266, and 10 (see Table 1 for strain names). Because this procedure was very time-consuming, the protocol was revised to determine the time for a culture to grow to about 5 times the original concentration (Percent-Growth Protocol for Algae). Early results showed that 121 and 123 (both *Chlamydomonas acidophila*) reached 500% growth after approximately 4 weeks and that 89, 266 (both *Chlorella*), and 10 (*Scenedesmus acutus f. alternans*) need less than 4 weeks to reach this level. All of these cultures will be retested to verify these results.

Strain name	Number	Taxonomic Division	Weeks to 5x growth
Ankistrodesmus convolutus	309	Chlorophyta	2 to 3
Ankistrodesmus sp.	547*	Chlorophyta	1 to 2
Chlamydomonas acidophila A.	121	Chlorophyta	4 *(retest)*
Chlamydomonas acidophila B.	123*	Chlorophyta	4 *(retest)*
Chlamydomonas applanata	120	Chlorophyta	2 to 3
Chlamydomonas reinhardtii A.	243	Chlorophyta	2 to 4
Chlamydomonas reinhardtii B.	244*	Chlorophyta	2
Chlorella fusca var. vacuolata	89	Chlorophyta	> 4 *(retest)*
Chlorella kessleri	266	Chlorophyta	> 4 *(retest)*
Chlorella vulgaris	90	Chlorophyta	1 to 2
Chlorococcum hypnosporum	178	Chlorophyta	*in progress*
Desmococcus sp.	548*	Chlorophyta	< 4
Nitzschia palea	160*	Bacillariophyta	*in progress*
Pseudokirchneriella subcapitata	517/37	Chlorophyta	2 to 4
Rhodomonas minuta	344*	Cryptophyta	X
Scenedesmus acutus f. alternans	8	Chlorophyta	2 to 3
Scenedesmus acutus f. alternans	10	Chlorophyta	> 4 *(retest)*
Ulothrix sp.	546*	Chlorophyta	< 4

Table 1: All of the strains that were frozen from November 7, 2003, to present are listed. The asterisk (*) beside the strain number indicates that the culture is bacterized while others are axenic. Also, the strain number, its taxonomic division, and the amount of time it takes for each strain to reach the desired 500% growth are also shown.

Thirteen additional strains were frozen producing the results shown in Table 1. Most of these were green algae belonging to the division Chlorophyta. To reach 500%

growth, *Ankistrodesmus* (309 and 547) needed 1 to 3 weeks, *Chlamydomonas* (120, 243, and 244) needed 2 to 4 weeks, *Chlorella* (90) needed 1 to 2 weeks, *Pseudokirchneriella subcapitata* (517/37) needed 2 to 4 weeks, and *Scenedesmus* (8) needed 2 to 3 weeks. *Ulothrix sp.* (546) and *Desmococcus sp.* (548) were very hard to quantify due to excessive clumping, but both needed less than 4 weeks to reach a level where the culture would be ready for distribution. *Chlorococcum hypnosporum* (178) has only been thawed once and is currently in the progress of growth, thus no results are yet available. The cryptophyte *Rhodomonas minuta* (344) has been frozen and thawed twice and both times no viable cells were observed.

Freshwater diatoms are difficult to cryopreserve but we successfully froze and revived *Nitzschia palea* (160) using a slightly different protocol. Quantifying the results, however, is problematic due to clumping but we have established that sufficient cells are viable to produce a new culture. Further details will be available at a later date.

Discussion

The primary objective of this cryopreservation project was to test for the viability of the cryopreserved cultures in liquid nitrogen. When testing for percent-viability, there were inaccuracies in the cell counts since cells were not distributed evenly throughout the cryovials. These inaccuracies were also the product of motile cells, which were hard to quantify, but if immobilized with Lugol's iodine became indistinguishable from dead cells. Due to time constraints, percent-viability testing was discontinued, as it is also very labour intensive.

Percent-Growth Protocol for Algae was initiated to determine how long it took for cultures to grow to approximately 5 times the original concentration (~$5x10^6$ cells/ml). This approach is more practical as it is less time consuming and enables us to freeze more strains with less time needed for estimation of the results. Information on the length of time necessary for the culture to grow to 5 times the original concentration allows us to estimate the length of time from thawing a culture to the date it could be shipped. At the present time, 18 strains have been frozen, of which 5 strains (121, 123, 89, 266, and 10) need to be retested.

The current protocol seems to work well with various types of unicellular green algae (Chlorophyta) but will need to be modified for other types of algae such as cryptomonads, (e.g. *Rhodomonas minuta*, 344) which are difficult to cryopreserve. Freezing tolerance may be induced in some strains by "limitation of nutrients, …addition of metabolic inhibitors and the use of hypertonic growth media" (Morris, 1981). Modifying our protocols according to these factors may allow us to freeze strains, which are recalcitrant under our current protocol. One modification that increased the growth rate of post-thaw cultures was keeping the tubes horizontal in direct light and gently agitating them instead of having them standing upright. This increased the surface area that was exposed to direct light and could reduce the time needed for the cultures to reach 500% growth.

As cultures can become contaminated during the various procedures, it is essential to take all the necessary precautions to avoid this by using good sterile technique. The laminar flow hood and the outside of the cryovials should be sprayed with 70% ethanol and the number of times of opening the culture containers must be minimized. One of the

ways in which minimal handling of cultures could be achieved, is by using a spectrophotometer to estimate the density of the culture by measuring its turbidity rather than withdrawing aliquots for counting. This would be especially useful in measuring the growth of colonial or clumping cultures such as *Desmococcus sp.* (548) and *Ulothrix sp.* (546). Further modification of the new protocol and testing for cryopreservation of *Nitzschia palea* (160) will also continue. Also, we will initiate cryopreservation of some of our strains of cyanobacteria using DMSO as a cryoprotectant.

Conclusions

Eventually, we would like to have most of the strains in UTCC collection preserved in the vapour phase of liquid nitrogen in order to reduce the amount of work and space required to maintain the cultures, virtually eliminate genetic drift and contamination, and most importantly, preserve them for the long term and make them accessible whenever needed. A good protocol has been developed for cryopreservation of unicellular green algae and thus can be applied to other unicellular green algae strains, which have yet to be frozen. The success rate was very high, as only one of the 18 tested strains did not survive the cryopreservation. Our next phase will include cryopreserving our cyanobacteria strains. The entire project will take several years and will be a valuable improvement to the UTCC facility.

Acknowledgments

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References

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Day J.G., Fleck R.A., Benson E.E. (2000) Cryopreservation-recalcitrance in microalgae: novel approaches to identify and avoid cryo-injury. J. Appl. Phycol. 12: 369-377.

Morris, G.J. (1981) Cryopreservation. An introduction to cryopreservation in culture collections. Institute of Terrestrial Ecology, Cambridge. 27pp.

Appendix I

CRYOTANK MONTHLY INVENTORY

DATE: _____

BOX #	RACK 1	RACK 2	RACK 3	RACK 4
1				
2				
3				
4				
5				
6				
7				
8				

Appendix II

CRYOTANK INVENTORY SYSTEM

Rack #: _____ Box #: _____ Date: _____

Frozen by: _____
Recovered by: _____

Comments:
_____
_____
_____

1	2	3	4	5	6	7	8	9
10	11	12	13	14	15	16	17	18
19	20	21	22	23	24	25	26	27
28	29	30	31	32	33	34	35	36
37	38	39	40	41	42	43	44	45
46	47	48	49	50	51	52	53	54
55	56	57	58	59	60	61	62	63
64	65	66	67	68	69	70	71	72
73	74	75	76	77	78	79	80	81

Appendix III

CRYO DATA SHEET

Date of freezing	
Name of species (number)	**( )**
Date of original stock culture	
Diluted stock concentration	**x 10^6 cells/ml**
Cryoprotectant	**5% MeOH**

Thawing after 1 week: DATE _____

COUNTS / **CULTURE**	**ORIGINAL []** ***(x 10^6 cells/ml)***	**WEEK 1 GROWTH % ( )**	**WEEK 2 GROWTH % ( )**	**WEEK 3 GROWTH % ( )**	**WEEK 4 GROWTH % ( )**
1					
2					
AVERAGES					
Date into full light:					
COMMENTS					

Thawing after 1 month: DATE _____

COUNTS / **CULTURE**	**ORIGINAL []** ***(x 10^6 cells/ml)***	**WEEK 1 GROWTH % ( )**	**WEEK 2 GROWTH % ( )**	**WEEK 3 GROWTH % ( )**	**WEEK 4 GROWTH % ( )**
1					
2					
AVERAGES					
Date into full light					
COMMENTS					