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# Cryopreservation of Eukaryotic Algae

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## ABSTRACT

Various cryoprotectants and cryopreservation techniques were applied to a wide selection of eukaryotic algal cultures. The most successful protocol involved algae cultured under low light (17-22$\mu$E $m^{-2}$ $s^{-1}$) on 1% agar media, controlled cooling at 1C $min^{-1}$ with 5% v/v $Me_2SO$ cryoprotectant, rapid thawing from -196C storage, and return to low light-1% agar media cultivation. This protocol allowed survival of 285 (78%) out of 365 algal strains tested. Moreover, 29 of the 57 genera were cryopreserved for the first time. Preliminary data suggest certain relationships among phylogenetic lines, habitats, cryoprotectants, and cryopreservability.

## INTRODUCTION

Cryopreservation of eukaryotic algae has been limited to relatively few taxa and has focussed mainly on damage sites induced by the freeze/thaw regime. Many of these studies aimed to understand freezing injury of higher plants or to store and maintain viable seed stocks (Gresshoff, 1977; Morris, 1981; Jordan et al., 1982; Styles et al., 1982; and Withers, 1987). Contrastingly, cryopreservation of algae as an alternative for maintaining algal cultures in a genetically stable state has not been a major goal. This goal has been all the more difficult, because poor understanding of the reactions between culture media, culture conditions, cryoprotectants, and organisms have kept cryopreservation studies of eukaryotic algae quite empirical.

Our objectives were to test a broader spectrum of algal taxa for cryopreservation; to investigate relationships between cryoprotectants, culture conditions, phylogenetic lineages, habitats, and cryopreservability; and to find a cryopreservation protocol offering a high degree of recovery.

## MATERIALS AND METHODS

### Cultures and Culturing

We used 365 algal strains representing 13 algal classes obtained from various sources and grown in several culture media. Culturing was in liquid and/or on 1% agar media at 20C under cool white fluorescent light of 17-22$\mu$E $m^{-2}$ $s^{-1}$. Liquid cultures were grown to densities of ca. $10^5$ cells $ml^{-1}$ in 15 to 20 ml of media in tubes.

### Cryoprotectants

Various concentrations of dimethylsulfoxide ($Me_2SO$), methanol, glycerol, dimethylsulfonium proprionate (DMSP), dimethylsulfide ($Me_2S$), betaine, polyvinylpyrroliodone (PVP-40, average mol wt 40,000), trehalose, and the mixtures $Me_2SO$ + 0.1M trehalose and methanol + 0.1M trehalose were tested for their overall effectiveness on a select few algae. Cryoprotectants were added to 2 ml plastic Nalgene screwcap cryovials containing a cell suspension of ca. $10^5$ cells $ml^{-1}$, final volume 1 ml. Cell suspensions were exposed to cryoprotectants < 45 min prior to initiation of freezing to ensure adequate penetration while minimizing toxic effects. An exception was the use of 0.1M trehalose as the sole cryoprotectant

which was exposed to cells 24 h prior to freezing, as shown necessary by Bhandal (1985).

### Freezing Cycle

A two-step cooling regime similar to that of McGrath (1979) was used throughout. After exposure to cryoprotectant, cryovials were placed in the freezing chamber of a Cryomed model 1010A programmable freezing controller. The chamber was cooled 1C $min^{-1}$ with rapid cooling (25C $min^{-1}$) around the heat of fusion point to reduce the length of the heat of fusion plateau. Subsequently, the chamber was warmed at 6C $min^{-1}$ until sample and chamber temperatures were within 6C of each other; then an immediate return to a cooling rate (chamber) of 1C $min^{-1}$ was initiated until the sample reached -40C. After 15 min at -40C, vials were plunged into liquid nitrogen (-196C).

### Thawing

After a minimum of 1 wk in liquid nitrogen, vials were removed and placed in a 40C circulating water bath until the last visible ice crystal had melted. Vials were then agitated to produce a homogenous cell suspension, 0.02 ml of which was placed on 3 replicate 60 X 15 mm Petri plates containing 15 ml of sterile 1% agar media and spread with a sterile glass rod. Dishes were sealed with Parafilm and incubated as above. A large body of data show that cryopreservation success is little influenced by cryostorage time from several days to several years for several different cell types (Matsuki et al., 1990; Morris, 1981; Schwartz et al., 1990; Simione et al., 1977; and Simon, 1972), and we have rechecked several of our replicate vials after 6-12 months with essentially the same results.

### Viability

All plates were checked for growth up to 25 days. Recovery rates were assessed by comparison of colony forming units (CFU) of freeze/thaw samples with similarly plated aliquots of the unfrozen cultures or to Coulter electronic particle counts of unfrozen cultures. Evaluation of viability was performed and scored as follows: NG for no growth, + for 1-100 CFU's, + + for 100-1000 CFU's, and + + + for >1000 CFU's. Accurate direct cell count studies proved the reliability of this scoring scale and showed that Coulter counting produced high estimates of viable cells. Successful recovery was defined as those cultures producing enough colonies to reclaim the culture (2% of the CFU's of control plates or > 0.2% of the Coulter electronic particle count due to its overestimation of viable cell counts).

## RESULTS

Our preliminary surveys with a smaller selection of algal cultures revealed that cells grown in low light (vs high) on agar media (vs liquid) with 5% $Me_2SO$ as cryoprotectant (vs other concentrations and other cryoprotectants) were cryopreserved more often than other combinations. The application of this more successful protocol to 365 strains is summarized in Table 1. The 365 strains comprised 68 genera, 33 not previously tested; 285 strains (78%) including 57 genera, of which 29 were heretofore untested, were recovered successfully. Comparison among algal classes which contained >10 strains (Bacillariophyceae, Chlorophyceae, Eustigmatophyceae, Prasinophyceae, and Xanthophyceae) indicated only slight variations in successful recovery. However, within an algal class

TABLE 1. List of 68 algal genera examined in this study with respect to their cryopreservation success given in # of strains successfully recovered and unsuccessfully recovered.

| Genera | No. of Strains Successfully Cryopreserved | No. of Strains Unsuccessfully Cryopreserved |
|---|---|---|
| Bacillariophyceae | | |
| *Achnanthes** | 1 | 0 |
| *Cylindrotheca** | 2 | 0 |
| *Navicula** | 2 | 0 |
| *Nitzschia* | 0 | 1 |
| *Phaeodactylum* | 5 | 0 |
| *Stauroneis** | 1 | 0 |
| Charophyceae | | |
| *Euastrum** | 0 | 1 |
| *Mesotaenium* | 0 | 1 |
| *Zygnema** | 0 | 1 |
| Chlorophyceae | | |
| *Ankistrodesmus* | 2 | 0 |
| *Asterococcus** | 1 | 0 |
| *Axilosphaera** | 1 | 0 |
| *Borodinellopsis** | 1 | 0 |
| *Bracteacoccus* | 3 | 0 |
| *Carteria** | 1 | 0 |
| *Characium* | 1 | 0 |
| *Chlamydomonas* | 52 | 39 |
| *Chlorella* | 20 | 1 |
| *Chlorococcum* | 2 | 0 |
| *Coccomyxa* | 13 | 1 |
| *Dactylococcus* | 1 | 0 |
| *Dunaliella* | 1 | 5 |
| *Eudorina* | 0 | 2 |
| *Gloeocystis** | 0 | 1 |
| *Gonium** | 0 | 2 |
| *Haematococcus* | 1 | 0 |
| *Nannochloris* | 1 | 2 |
| *Neochloris** | 5 | 0 |
| *Neospongiococcum** | 2 | 0 |
| *Oocystis* | 1 | 0 |
| *Palmella** | 1 | 0 |
| *Pandorina** | 0 | 2 |
| *Planophila** | 1 | 0 |
| *Platymonas** | 2 | 0 |
| *Pseudococcomyxa* | 1 | 0 |
| *Pseudotetracystis** | 1 | 0 |
| *Scenedesmus* | 8 | 1 |
| *Tetracystis* | 1 | 0 |
| *Trebouxia* | 1 | 0 |

*continued*

TABLE 1. *continued*

| Genera | No. of Strains Successfully Cryopreserved | No. of Strains Unsuccessfully Cryopreserved |
|---|---|---|
| Cryptophyceae | | |
| *Cryptomonas* | 0 | 1 |
| Cyanophyceae | | |
| *Agmenellum** | 1 | 0 |
| *Calothrix* | 1 | 0 |
| *Lyngbya* | 0 | 1 |
| *Nostoc* | 1 | 0 |
| Dinophyceae | | |
| *Crypthecodinium* | 1 | 0 |
| Euglenophyceae | | |
| *Euglena* | 0 | 2 |
| Eustigmatophyceae | | |
| *Eustigmatos** | 2 | 0 |
| *Mondus* | 0 | 1 |
| *Nannochloropsis* | 6 | 2 |
| *Pleurochloris* | 1 | 0 |
| *Vischeria* | 4 | 0 |
| Phaeophyceae | | |
| *Ectocarpus** | 0 | 2 |
| *Sphacelaria** | 0 | 1 |
| Prasinophyceae | | |
| *Prasinocladus** | 2 | 0 |
| *Pyramimonas** | 1 | 0 |
| *Tetraselmis** | 101 | 7 |
| Prymnesiophyceae | | |
| *Ochrosphaera** | 0 | 1 |
| Rhodophyceae | | |
| *Porphyridium* | 3 | 0 |
| Xanthophyceae | | |
| *Botrydiopsis** | 3 | 0 |
| *Botrydium* | 2 | 0 |
| *Bumilleria* | 1 | 1 |
| *Bumilleriopsis** | 1 | 0 |
| *Chlorellidium** | 1 | 0 |
| *Chloridella* | 1 | 1 |
| *Chlorocloster** | 7 | 0 |
| *Heterococcus** | 4 | 0 |
| *Heterothrix** | 4 | 0 |
| *Nephrodiella* | 1 | 0 |

* indicates those genera which have not previously been examined

recovery rates varied markedly. The cryopreservation success rate for the algal orders tested within the Chlorophyceae is summarized in Table 2. The Chlorellales, Chlorococcales, and Chlorosarcinales which included many edaphic taxa showed much higher recovery rates than the Tetrasporales or Volvocales which included mainly aquatic taxa.

Percent recoveries of six algal taxa, representing xanthophycean and chlorophycean lineages (Dodge, 1979), frozen in optimal concentrations of 5 cryoprotectants are summarized in Table 3. The two lineages reacted differently to the various cryoprotectants. However, organisms within the same phylogenetic lineage exhibited similar responses to a given cryoprotectant. For example, the recovery rates of chlorophycean and prasinophycean algae decreased with increased concentrations of betaine, while recovery rates of bacillariophycean, eustigmatophycean, and xanthophycean algae were enhanced by increased concentrations of this compound.

## DISCUSSION

Our studies, aimed at developing a better cryopreservation protocol applicable to a broader spectrum of eukaryotic algae, have continued with a largely empirical approach. However, an improved understanding and some intriguing ideas have emerged. Growth under relatively low light (17-22 $\mu$E $m^{-2}$ $s^{-1}$) and on agar media may have increased survivability by preconditioning the cells and reducing the amount of intracellular vacuolation, as suggested earlier by Morris (1981). Our recovery rates among algal classes showed only slight variations suggesting that taxonomic classes do not vary in the cryopreservability of their respective members; that is, class differences do not seem important to cryopreservation success. However, within the Chlorophyceae, the higher rates of success seen in the Chlorellales, Chlorococcales, and Chlorosarcinales as compared to the Tetrasporales and Volvocales suggest habitats may play a role in determining cryopreservability. The higher success rates of the first three orders above may be due to cytological or physiological mechanisms that counteract the hydration/dehydration and freeze/thaw cycles typical of many soil environments inhabited by many taxa from these groups (Round, 1981). Conversely, the more highly vacuolated Tetrasporales and Volvocales would not be expected to withstand desiccation- or freeze-induced stresses as well in their vegetative state. Their typical aquatic habitat lacks the desiccation and rapid temperature changes or freeze stresses of many soils. Therefore, development of desiccation- and freeze-resistant properties may be poorly developed in most of these aquatic species, except when they produce resting spores.

Recovery rates of several algae from two different phylogenetic lineages were compared (Table 3). The difference in reactions of these two taxonomic groupings to the various cryoprotectants and the similarity of reaction of the organisms within a given lineage may be attributable to conserved physiological characteristics along these phylogenetic lines. More research is needed for validation of such trends, which if confirmed, may allow selection of a cryoprotectant for a given organism and a better understanding of the cryoprotectant action.

This survey of 365 algal strains is the largest to date and encompasses more algal taxa than have been previously examined. The success rate of 78% recovery of all the algal strains tested rivals the success rates of the prokaryotes which includes

TABLE 2. Success rate of cryopreservation of orders within the algal class Chlorophyceae using 5% (v/v) dimethylsulfoxide as the cryoprotectant. % in ( ) indicate <10 strains tested.

| Order | No. of Strains Successfully Cryopreserved | No. of Strains Unsuccessfully Cryopreserved | % Success |
|---|---|---|---|
| Chlorellales | 39 | 4 | 90.7 |
| Chlorococcales | 21 | 1 | 95.5 |
| Chlorosarcinales | 5 | 0 | (100.0) |
| Tetrasporales | 2 | 1 | (66.6) |
| Volvocales | 58 | 50 | 53.7 |

TABLE 3. Percent recoveries based on CFU's and Coulter electronic particle counts of 6 algal taxa from two taxonomic lineages. % in ( ) are calculated from the electronic particle counts. NG denotes no growth.

| TAXA | 5% (v/v) $Me_2SO$ | 10% (v/v) Glycerol | 10% (v/v) Methanol | 1% (w/v) DMSP | Betaine 1% | Betaine 5% | Betaine 10% |
|---|---|---|---|---|---|---|---|
| | | | XANTHOPHYCEAN LINE | | | | |
| Bacillariophyceae | | | | | | | |
| *Achnanthes* | (3.3) | (0.3) | (0.13) | (NG) | (0.2) | (1.9) | (1.4) |
| *brevipes* | 18 | 26 | 13 | NG | 2.9 | 27 | 19.7 |
| Eustigmatophyceae | | | | | | | |
| *Eustigmatos* | (11.2) | (39.4) | (9.9) | (1.3) | (2.1) | (4.1) | (29.5) |
| *vischeri* | 18.6 | 53.2 | 14.4 | 2.8 | 3.15 | 6.17 | 44.5 |
| Xanthophyceae | | | | | | | |
| *Heterococcus* | (53.9) | (24.4) | (14.8) | (1.2) | (29.9) | (50.8) | (55.9) |
| *fuornensis* | 96.9 | 63.5 | 40.4 | 2.95 | 50 | 84.8 | 93.4 |
| | | | CHLOROPHYCEAN LINE | | | | |
| Prasinophyceae | | | | | | | |
| *Tetraselmis* | (1.6) | (2.7) | (0.3) | (NG) | (2.1) | (1.2) | (1.0) |
| sp. 87 | 2.74 | 7.0 | 1.04 | NG | 7.5 | 4.2 | 3.6 |
| Chlorophyceae | | | | | | | |
| *Chlamydomonas* | (18) | (23.6) | (38.7) | (13) | (27.7) | (19.6) | (19) |
| *pseudococcum* | 105 | 44.3 | 81.3 | 30.9 | 62.8 | 44.5 | 42.8 |
| *Chlorella* | (27.4) | (27.8) | (34.7) | (0.08) | (64.3) | (58.4) | (29.2) |
| *vulgaris* | 112 | 31.7 | 54.3 | 0.14 | 147 | 134 | 67 |

the cyanobacteria (Albrecht et al., 1973; Heckly, 1978). This study shows that cryopreservation can be used as an alternative for maintaining cultures of eukaryotic algae and, in addition, for maintaining genetic stability and strain integrity. More studies are needed to determine the influence of habitats and phylogenetic lineages on algal cryopreservability, as well as to seek new cryoprotective compounds such as betaine.

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