VIABILITY OF A PHYTOFLAGELLATE AFTER FREEZING

J. P. Flassch, G. Salaun, Y. Normant
Centre Océanologique de Bretagne, B. P. 337
29273 Brest, France

ABSTRACT

The Prasinophyceae Tetraselmis suecica Butcher, was cultivated at a density of 2 to 3 million cells per ml and was concentrated with a decanting centrifugal extractor at 1 m³/hr flow. The samples, obtained in the form of a paste, were diluted to 50 x 10⁶ cells per ml and mixed with two cryoprotective agents such as glycerol (10, 20, 30%) and dimethyl sulfoxide (DMSO, 5, 10, 15%), then frozen and stored at -25 C.

After a 1 month period, results by decreasing order of viability are as follows: glycerol, 10%, 20%, DMSO 15%, glycerol 10%, a starting culture of 0.69 x 10⁶ cells per ml in 500 ml sea water enriched with Conway stock solution reached 5.49 x 10⁶ cells per ml culture at the end of 4 days.

The aim of these experiments was to obtain a good conservation of phytoflagellate "master cultures" and to store seed cultures so that they could be distributed to "field hatcheries", eliminating the need for them to produce their own seed cultures which must be voluminous in order to obtain the rapid and efficient growth of the necessary large volume cultures.

INTRODUCTION

This research on the viability of a marine phytoflagellate is the result of the increasing requirements at the heart of the aquaculture research unit. At first, we thought it fit to produce a

---

1Contribution no. 337 du Département Scientifique du Centre Océanologique de Bretagne.
basic phytoplanktonic food of which the principal alga was Platy-
monas suecica, Kylin (Tetraselmis succica, Butcher) (Parke and Dixon,
1968). This food gave good results in growth of herbivores such as
oysters or abalone and also with Brachiomus plicatilis and Artemia
salina, these two species being used as the basic food for shrimp
and fish larvae. With increasing requirements, the unit had to pro-
duce much more of this alga.

The handling of many m³ per day, with at least 10⁶ cells per
ml, obliged us to tackle the technology for concentrating this
species. This problem was resolved with a decanting centrifugal
extractor. With this method, in the best periods of the year, the
unit produced many possible concentrates of the culture up to and
including paste form.

Storage of surplus algae paste required freezing. Since tests
showed this paste to be viable as seed culture material, tests were
conducted to determine the viability of the paste culture after
freezing. The study had two aims: 1) improving storage of "master
cultures" (Holm-Hansen, 1973) to eliminate the harmful effects of
handling, and 2) utilizing the frozen and thawed samples as seed
cultures to produce large volume cultures sufficient for feeding pur-
poses, eliminating the need for breeding enterprises to prepare
their own large volume seed cultures.

Studies on the survival of freshwater algal cultures, with
experiments on flagellates (Hwang and Honneland, 1965), and studies
on algae from the Antarctic (Holm-Hansen, 1963) show that cryopro-
tective agents are very useful in decreasing cell mortality during
freezing and thawing.

MATERIALS AND METHODS

Preparation of Samples

We started from new concentrated cells at 20 C in fresh pasté
obtained at 3000 revolutions a minute and at the flow rate of 1 m³
per hour and resuspended to 50 x 10⁶ cells/ml in 20 C sea water.

Two cryoprotective agents were mixed with the samples at vari-
ous concentrations: glycerol at 10%, 20% and 30% and dimethyl
sulfoxide (DMSO) at 5%, 10% and 15% (Table 1).

Each 10 ml sample contained approximately 5 x 10⁶ cells. The
container used for storage was a 50 ml plastic box. The time
required for preparation of this sample from the culture before
concentration was about 30 minutes.

Freezing and Thawing

The samples were put in a freezing chamber at -25 C and stored
in this place without controlled cooling. After being frozen for 5, 11, 25 and 32 days, samples of the frozen material were slowly thawed at 20°C.

The Starting Culture

After the thawing of each recovered sample, 5 ml were resuspended in 500 ml of sea water with 0.5 ml Conway stock solution (Walne, 1966) in a liter flat glass flask, previously sterilized. Vitamins were supplied after sterilization. The number of cells at the starting point varied with the concentration of the protective agent. Cultures were stirred by aeration (air + 1% CO₂).

Determination of Viability

Viability was determined by the total cell counts per ml after 2 to 4 hours, 48 to 52 hours, and 72 to 77 hours. An estimation of the motility and the mitotic activity was determined by an arbitrary scale (Table 1).

RESULTS

Cell counts from samples frozen for 5, 11, 25, and 32 days are presented in Table 1 (error ± 12%). Records of the population levels after incubation at various intervals are also included with a scale of cellular motility and mitotic activity.

The best results were obtained (in decreasing order of viability) with glycerol 10%, 20%, DMSO 15%, glycerol 30%, DMSO 10%, 5%.

DISCUSSION

The choice of freezing temperature was due to the fact that we noted a certain liveliness in the cells after 24 hours of -25°C freezing despite the lack of a cryoprotective agent, which suggested the resistance of this species to the cold. For this reason, and in order to simplify the operations, a regular freezer seemed the most practical.

In fact, the conservation of a paste which will later be diluted in 60 to 80 liters of water needs space (0.5 to 1 liter) but not too long a time of conservation. In this case it would be difficult and expensive to maintain containers of liquid nitrogen (-160 to -196°C) in order to allow the conservation of voluminous samples.

Viability as indicated by motility and mitotic activity was high with the protective agent, glycerol 10% and 20%. When this agent was used, more than 20% of motile and dividing cells were
noted at 72 hours after thawing without apparent differences with regard to the duration of freezing. On the other hand, we observed a deterioration of viability with respect to duration of freezing when DMSO was used as the protective agent.

After a week of freezing, there appears to be a delay of 24 hours before the cells become motile.

It is difficult to get great precision in the determination of the index of motility. Nevertheless this index gives an indication of the evolution of the culture. The error on the other data, with single and dividing cells, is about 12% (determined from three cell counts).

The cell counts presented in Table 1 were sometimes lower at 48 hours than at 2 hours when the culture started badly. This difference was due to the error and to the fact that the cells settled in the flasks. It is difficult to know exactly how many cells are killed by freezing and to determine the portions of the multiplication which come from old cells and from new cells.

Nevertheless in the case of DMSO 15% and 10% and also sometimes glycerol 20%, we observed cultures with a good concentration of cells at 72 hours but which contained many packets of dead cells without flagellae. In this case, the culture was not suitable to be used as a seed culture. This inefficient culture is the result of a bad protection. It is probable that we can get better results with a freezing temperature around -40 C and storage at -25 C.

CONCLUSION

It seems obvious that this species of high nutritive quality, when protected by a 10% concentration of glycerol, is able to live, to move, and to multiply after thawing at a constant temperature (20 C), having been frozen and stored for at least 1 month at -25 C.

We must now try to improve these results in order to inoculate volumes of 60-80 liters at 0.5 x 10^6 cells per ml in order to obtain a culture of 2 x 10^6 cells per ml in 3-4 days.

Once this stage has been reached, it will be feasible to store the seed cultures protected by a cryoprotective agent and distribute them within a month's time to field hatcheries or breeding enterprises which are not able to use anything except flagellate algae such as P. suecica.

LITERATURE CITED


Table 1. Viability of *Platymonas suecica* after freezing

<table>
<thead>
<tr>
<th>Cryoprotective agents</th>
<th>Hours of restarted culture</th>
<th>Time of freezing in days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N x 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>Glycerol 20%</td>
<td>2</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td>DMSO 10%</td>
<td>2</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2.16</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
</tr>
</tbody>
</table>

Coefficients: 1 < 1% of motile or dividing cells

N x 10^6 = Number of cells x 10^6
M = Motility
MA = Mitotic activity