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# VIABILITY OF A PHYTOFLAGELLATE AFTER FREEZING[1]

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## 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 $m^3$/hr flow. The samples, obtained in the form of a paste, were diluted to 50 x $10^6$ 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 $10^6$ cells per ml in 500 ml sea water enriched with Conway stock solution reached 5.49 x $10^6$ 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

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basic phytoplanktonic food of which the principal alga was Platymonas 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 Brachionus 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 produce much more of this alga.

The handling of many $m^3$ per day, with at least $10^6$ 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 purposes, 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 cryoprotective 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 paste obtained at 3000 revolutions a minute and at the flow rate of 1 $m^3$ per hour and resuspended to 50 x $10^6$ cells/ml in 20 C sea water.

Two cryoprotective agents were mixed with the samples at various 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 $10^6$ 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% $CO_2$).

### 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 $0.5 \times 10^6$ cells per ml in order to obtain a culture of $2 \times 10^6$ 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

Hwang, S. W., and W. Honneland. 1965. Survival of algal cultures after freezing by controlled and uncontrolled cooling. Cryobiology 1:305-311.

Holm-Hansen, O. 1963. Viability of blue-green and green algae after freezing. Physiological Plant 16:530-540.

Holm-Hansen, O. 1973. Preservation by freezing and freeze-drying. Handbook of Phycological Methods, Culture Methods and Growth Measurements (J. R. Stein, ed.). Cambridge University Press. 448 pp.

Parke, M., and P. S. Dixon. 1968. Check list of British marine algae second revision. Journal Marine Biological Association U. K. 48:783-832.

Walne, P. R. 1966. Experiments in the large scale culture of the larvae of Ostrea edulis. Fishery invest. Lond., Ser. 2 25(4):1-53.

Table 1. - Viability of <u>Platymonas suecica</u> after freezing

| Cryoprotective agents | | Hours of restarted culture | Time of freezing in days: 5 | | | 11 | | | 25 | | | 32 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | N x $10^6$ | M | MA | N x $10^6$ | M | MA | N x $10^6$ | M | MA | N x $10^6$ | M | MA |
| Glycerol % | 10 | 2 | 0.55 | 5 | - | 0.59 | 0 | 0 | 0.60 | 0 | 0 | 0.69 | 0 | 5 |
| | | 48 | 1.03 | 7 | - | 0.69 | 5 | 7 | 0.74 | 6 | 4 | 0.95 | 7 | 5 |
| | | 72 | - | - | - | 2.89 | 3 | 6 | 4.51 | 5 | 0 | 2.73 | 4 | 4 |
| | 20 | 2 | 0.60 | 5 | - | 0.70 | 0 | 0 | 0.55 | 0 | 0 | 0.63 | 0 | 4 |
| | | 48 | 2.16 | 7 | - | 0.59 | 5 | 5 | 0.87 | 4 | 5 | 0.81 | 1 | 5 |
| | | 72 | - | - | - | 1.01 | 4 | 5 | 2.63 | 5 | 4 | 2.53 | 1 | 5 |
| | 30 | 2 | 0.50 | 0 | - | 0.98 | 0 | 0 | 0.50 | 0 | 0 | 0.63 | 0 | 4 |
| | | 48 | 0.46 | 3 | - | 0.66 | 4 | 4 | 0.69 | 0 | 4 | 0.59 | 0 | 2 |
| | | 72 | - | - | - | - | - | - | 0.69 | 1 | 4 | 0.71 | 0 | 0 |
| DMSO % | 5 | 2 | 0.65 | 1 | - | 0.90 | 0 | 0 | 0.90 | 0 | 0 | 0.69 | 0 | 2 |
| | | 48 | 0.87 | 4 | - | 1.10 | 1 | 3 | 0.92 | 0 | 0 | 0.79 | 0 | 0 |
| | | 72 | - | - | - | 0.90 | 4 | 5 | 0.70 | 1 | 0 | - | - | - |
| | 10 | 2 | 0.60 | 4 | - | 0.72 | 0 | 0 | 0.60 | 0 | 0 | 0.63 | 0 | 3 |
| | | 48 | 1.10 | 4 | - | 0.73 | 4 | 4 | 0.54 | 1 | 0 | 0.68 | 0 | 3 |
| | | 72 | - | - | - | 0.90 | 4 | 5 | 0.70 | 1 | 0 | - | - | - |
| | 15 | 2 | 0.62 | 5 | - | 0.73 | 0 | 0 | 0.55 | 0 | 0 | 0.73 | 0 | 4 |
| | | 48 | 1.38 | 7 | - | 0.69 | 4 | 4 | 0.64 | 1 | 0 | 0.89 | 0 | 1 |
| | | 72 | - | - | - | 1.92 | 3 | 6 | 0.73 | 1 | 0 | 0,88 | 5 | 6 |

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

Coefficients:
1 < 1% of motile or dividing cells
1 < 2 < 2%
2 < 3 < 4%
4 < 4 < 10%
10 < 5 < 20%
20 < 6 < 25%
25 < 7