Effect of Growth Temperature on the Cryopreservation of *Prototheca*

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SUMMARY

The temperature at which *Prototheca* spp. were grown determined their response to freezing to -196°C and subsequent thawing. Cells cultured at 35°C were the most sensitive to freezing injury; at lower growth temperatures, resistance to freezing damage was seen. At all culture temperatures examined, the freezing tolerance varied with the age of the culture.

INTRODUCTION

Prototheca is a unicellular, colourless heterotrophic organism which has been classified as an apoplastidic relation of the genus *Chlorella* (Cooke, 1968). On the basis of nutritional similarities (Pore, 1972) and cell-wall polysaccharide composition (Conte & Pore, 1973), *Prototheca* spp. have been shown to be related to *Chlorella protothecoides*, the only species of the sub-genus *Auxenochlorella* (Shihira & Krauss, 1965).

As *Prototheca* may be a pathogen of man and other animals (Emmons, Chapman & Utz, 1970), cultures were maintained under liquid nitrogen rather than by serial passage. The advantages of the cryopreservation of algal cultures in maintaining the original properties of organisms and in reducing the possibility of contamination have already been described (Holm-Hansen, 1963, 1971; Hwang & Horneland, 1965).

In preliminary experiments, *Prototheca* spp. were found to be sensitive to the stress of freezing to and thawing from -196°C . It was therefore decided to determine whether *Prototheca* could be made tolerant of freezing by cold acclimatization as can *C. fusca* var. *vacuolata* (Morris, 1976*b*), benthic seaweeds (Bird & McLachlan, 1974; Lyutova *et al.*, 1967) and higher plants (reviewed by Levitt, 1972). It has also been reported that the recovery following freezing and thawing of *Escherichia coli* (Toyokawa & Hollander, 1956), *Chlorella* spp. (Morris, 1976*a, b*) and *Tetrahymena* (Osborne & Lee, 1975) varies with the age of the culture. Because of the different growth rates of *Prototheca* at the experimental temperatures studied, it was necessary to determine tolerance of freezing on successive days of culture. As survival following freezing and thawing is related to the rate of cooling (reviewed by Mazur, 1970), the effect of rates of cooling to -196°C on cells grown at different temperatures was examined. Finally, the method of preservation developed for one strain of *Prototheca* was applied to seven other strains.

METHODS

Cells. All strains of *Prototheca* were obtained from the Culture Centre of Algae and Protozoa (CCAP), whose system of numbering is employed. Cells of *P. chlorellioides* CCAP263/1 were used to develop preservation techniques.

Cell culture. Cells were grown in 1 % (w/v) tryptone-1 % (w/v) glucose broth. Conical

flasks (100 ml) were inoculated with 1 ml of a stationary phase culture grown for 7 to 10 days, at 25 °C, and incubated in the dark.

Freezing and thawing. Cells from liquid culture were used without further preparation. The cell suspension (1 ml) was placed in 12 × 35 mm sterile polypropylene tubes (Sterilin, Richmond, Surrey), cooled at different rates to -60 °C using the technique previously described (Morris & Farrant, 1972), and then cooled rapidly to -196 °C in liquid nitrogen. The cooling rate was determined between -5 and -60 °C using a separate sample with a copper-constantan thermocouple (28 s.w.g.) connected to a Kipp-Zonen potentiometric recorder (Model BD5). Cells were thawed by rapid agitation of the tube in a water bath at 25 °C until all ice had melted.

Thermal shock. Samples of cells (1 ml) in polypropylene tubes were incubated in a water bath at 25 °C for 30 min and then placed in a stirred ice-water bath for 15 min; this produced an exponential and reproducible rate of cooling.

Viability assay. Samples of exponential dilutions of the cells (1 ml) in tryptone-glucose medium were pipetted into sterile Petri dishes (90 mm), and 25 ml of 1.5 % (w/v) agar in tryptone-glucose medium (40 to 42 °C) was added. After the cells had been dispersed by gentle agitation, the agar was allowed to solidify, and the plates were inverted and incubated at 25 °C. After 72 h, colonies were counted. The efficiency of plating for unfrozen cells, as determined by haemocytometer counts, was always greater than 95 %.

Five replicates were examined for each treatment.

RESULTS

Effect of growth temperature

Flasks were inoculated with *P. chlorelloides* and incubated at 4, 15, 25 or 35 °C. On successive days of culture, the cells were cooled to 0 °C or to -196 °C. Two methods of cooling to -196 °C were examined: rapid cooling, at a rate of 212 ± 17 °C min⁻¹, by direct immersion of the tube in liquid nitrogen ($N = 54$); and slow cooling, at a rate of 0.314 ± 0.011 °C min⁻¹, to -60 °C followed by transfer to liquid nitrogen ($N = 48$).

Under no experimental condition was there a loss of viability upon reduction in temperature from 25 to 0 °C (thermal shock). This is in contrast to the report that *C. protothecoides* in the exponential phase of growth is susceptible to thermal shock damage (Morris, 1976a).

Prototheca chlorelloides did not survive rapid cooling to -196 °C (recovery < 0.1 %) following growth at 25 or 35 °C (Fig. 1). However, cells cultured at lower temperatures showed a small but significant increase in recovery (Fig. 2). Following the slower rate of cooling, viable cells were recovered upon thawing from all cultures examined (Figs. 1 and 2). Decreasing the temperature of culture led to an increase in resistance to freezing and thawing. These results agree with those obtained for other algae (Bird & McLachlan, 1974; Lyutova *et al.*, 1967; Morris, 1976b) and higher plants (Levitt, 1972). At all experimental temperatures, freezing tolerance varied with the age of the culture (Figs. 1 and 2).

Effect of rate of cooling

Prototheca chlorelloides was cultured at 4, 15, 25 and 35 °C for periods, determined from Figs. 1 and 2, which gave cells with maximum freezing tolerance at each temperature. The effect on these cells of the rate of cooling to -196 °C was then determined. With all cultures, the highest recovery was obtained after the slowest cooling rate (0.3 °C min⁻¹); recovery

Fig. 1. Recovery of *P. chlorelloides* following freezing and thawing from -196 °C on different days of culture at (a) 35 °C or (b) 25 °C. Cells were cooled at a rate of 0.3 °C min⁻¹ (●) or 212 °C min⁻¹ (■). Growth curves of unfrozen control cells are included (○). In this and subsequent figures, standard errors are not given, but were always less than ± 2 % recovery.

Fig. 2. Recovery of *P. chlorelloides* following freezing and thawing from -196 °C on different days of culture at (a) 15 °C or (b) 4 °C. Cells were cooled at a rate of 0.3 °C min⁻¹ (●) or 212 °C min⁻¹ (■). Growth curves of unfrozen control cells are included (○).

decreased as the rate increased (Fig. 3). At all cooling rates studied, the recovery decreased as the temperature of the culture increased.

Cryopreservation of other *Prototheca* spp.

From the results illustrated in Figs. 1 to 3, it was decided that the best method for routine cryopreservation of *Prototheca* spp. was as follows. Cells were grown for 14 days at 4 °C, cooled at a rate of 0.3 °C min⁻¹ to -60 °C and then transferred to liquid nitrogen. The recoveries of viable cells of eight strains of *Prototheca* preserved by this technique are given

Fig. 3. Recovery of *P. chlorelloides* after cooling at different rates to -196°C . Cells were cultured for 7 days at 35°C (○), 3 days at 25°C (●), and for 14 days at 15°C (□) or 4°C (■).

Table 1. Recovery following freezing and thawing of eight strains of *Prototheca*

Cells were grown at 4°C for 14 days, cooled at a rate of $0.3^{\circ}\text{C min}^{-1}$ to -60°C and then transferred to liquid nitrogen for 7 days.

CCAP strain no.	Species	$10^{-5} \times \text{no. of cells/ml before freezing}$	Recovery (%)
263/1	<i>P. chlorelloides</i>	23	89
263/2	<i>P. moriformis</i>	43	84
263/3a	<i>P. portoricensis</i>	2.7	34
263/3b	<i>P. portoricensis</i>	2.5	62
263/4	<i>P. portoricensis</i> var. <i>trispurus</i>	3.4	46
263/5	<i>P. zopfii</i>	1.1	37
263/6	<i>P. krugeri</i>	1.5	48
263/7	<i>Prototheca</i> sp.	3.5	68

in Table 1. With all strains, the recovery upon thawing was greater than 30 % and the number of viable cells recovered/ml exceeded 10^4 , sufficient inoculum for a new culture. Long-term stability studies on these frozen samples are now in progress.

DISCUSSION

Previous studies on freezing tolerance at sub-optimal growth temperatures have been confined to photosynthetic organisms. Our results show that a similar cold acclimatization process occurs in the apoplastidic alga *Prototheca*.

The biochemical mechanism of this increase in freezing tolerance is not fully understood (reviewed by Levitt, 1972). It is generally assumed that cellular membranes are the primary sites of damage during freezing and thawing. Membrane lipids change from a liquid crystalline to a gel structure upon reduction in temperature (reviewed by Grant, 1975). The extent of these phase separations and the temperature at which they occur depend both on the lipid composition and the degree of fatty-acid saturation of the membrane (James & Branton, 1973; Schechter, Letellier & Gulik-Krzywicki, 1974). It has been suggested that the increase in the degree of unsaturation of membrane fatty acids at low growth temperatures in bacteria (Marr & Ingram, 1962; Farrell & Rose, 1967), *Chlorella* (Patterson, 1970) and higher plants (Gerloff, Richardson & Stahmann, 1966; Siminovitch *et al.*, 1968; Kuiper, 1970), or the increase in total membrane lipids at low growth temperatures (Singh, de la Roche & Siminovitch, 1975; Morris, 1976b) may be responsible for the cryoprotective effect.

This process of cold acclimatization provides a simple method for cryopreservation of

Prototheca without the addition of cryoprotective compounds. Cells may be used directly upon thawing without washing and centrifuging. Application of this technique as a general method for the cryopreservation of algae and protozoa is now in progress.

REFERENCES

- BIRD, C. J. & McLACHLAN, J. (1974). Cold-hardiness of zygotes and embryos of *Fucus* (Phaeophyceae, Fucales). *Phycologia* **13**, 214–225.
- CONTE, M. V. & PORE, R. S. (1973). Taxonomic implications of *Prototheca* and *Chlorella* cell wall polysaccharide characterization. *Archives of Microbiology* **92**, 227–233.
- COOKE, W. B. (1968). Studies in the genus *Prototheca*. 1. Literature review. *Journal of Elisha Mitchell Scientific Society* **84**, 213–216.
- EMMONS, C. W., CHAPMAN, C. H. & UTZ, J. P. (1970). *Medical Mycology*. Philadelphia, Pennsylvania: Lea & Febiger.
- FARRELL, J. & ROSE, A. H. (1967). Temperature effects on micro-organisms. In *Thermobiology*, pp. 142–169. Edited by A. H. Rose. London: Academic.
- GERLOFF, E. D., RICHARDSON, T. & STAHMANN, M. A. (1966). Changes in fatty acids of alfalfa roots during cold hardening. *Plant Physiology* **41**, 1280–1284.
- GRANT, C. W. M. (1975). Lipid lateral phase separations; spin label and freeze-fracture electron microscopy studies. *Biophysical Journal* **15**, 949–952.
- HOLM-HANSEN, O. (1963). Viability of blue-green and green algae after freezing. *Physiologia plantarum* **16**, 530–540.
- HOLM-HANSEN, O. (1971). Preservation by freezing and freeze-drying. In *Phycological Methods*, pp. 195–205. Edited by J. R. Stein. Cambridge: University Press.
- HWANG, S. & HORNELAND, W. (1965). Survival of algal cultures after freezing by control and uncontrolled cooling. *Cryobiology* **1**, 305–311.
- JAMES, R. & BRANTON, D. (1973). Lipid and temperature-dependent structural changes in *Acholeplasma laidlawii* cell membranes. *Biochimica et biophysica acta* **323**, 378–390.
- KUIPER, P. J. C. (1970). Lipids in alfalfa leaves in relation to cold hardiness. *Plant Physiology* **45**, 684–686.
- LEVITT, J. (1972). *Responses of plants to environmental stresses*. New York and London: Academic.
- LYUTOVA, H. I., ZAVADSKAYA, I. G., LUKINSKAYA, N. F. & FELDMAN, N. L. (1967). Temperature adaption of cells of marine and freshwater algae. In *The Cell and Environmental Temperature*, pp. 166–172. Edited by A. S. Troshin. Oxford: Pergamon.
- MARR, A. G. & INGRAM, J. L. (1962). Effect of temperature on the composition of fatty acids in *Escherichia coli*. *Journal of Bacteriology* **84**, 1260–1267.
- MAZUR, P. (1970). Cryobiology: The freezing of biological systems. *Science, New York* **168**, 939–949.
- MORRIS, G. J. (1976a). The cryopreservation of *Chlorella*. 1. Interactions of rate of cooling, protective additive and warming rate. *Archives of Microbiology* **107**, 57–62.
- MORRIS, G. J. (1976b). The cryopreservation of *Chlorella*. 2. Effect of growth temperature on freezing tolerance. *Archives of Microbiology* (in the Press).
- MORRIS, G. J. & FARRANT, J. (1972). Interactions of cooling rate and protective additive on the survival of washed human erythrocytes frozen to -196°C . *Cryobiology* **9**, 173–181.
- OSBORNE, J. A. & LEE, D. (1975). Studies on conditions required for optimum recovery of *Tetrahymena pyriformis* strain s (phenoset A) after freezing to and thawing from -196°C . *Journal of Protozoology* **22**, 233–236.
- PATTERSON, G. W. (1970). Effect of culture temperature on fatty acid composition of *Chlorella sorokiniana*. *Lipids* **5**, 597–600.
- PORE, R. S. (1972). Nutritional basis for relating *Prototheca* and *Chlorella*. *Canadian Journal of Microbiology* **18**, 1175–1177.
- SHECHTER, E., LETELLIER, E. & GULIK-KRZYWICKI, T. (1974). Relations between structure and function in cytoplasmic membrane vesicles isolated from an *Escherichia coli* fatty-acid auxotroph. High-angle X-ray diffraction, freeze etch electron microscopy and transport studies. *European Journal of Biochemistry* **49**, 61–76.
- SHIHARA, I. & KRAUSS, R. W. (1965). *Chlorella: Physiology and Taxonomy of Forty-one Isolates*. College Park, Maryland: University of Maryland Press.
- SIMINOVITCH, D., RHEAUME, B., POMEROY, K. & LEPAGE, M. (1968). Phospholipid, protein and nucleic acid increases in protoplasm and membrane structures associated with development of extreme resistance in black locust tree cells. *Cryobiology* **5**, 202–225.
- SINGH, J., DE LA ROCHE, I. A. & SIMINOVITCH, D. (1975). Membrane augmentation in freezing tolerance of plant cells. *Nature, London* **257**, 699–700.
- TOYOKAWA, K. & HOLLANDER, D. H. (1956). Variation in sensitivity of *Escherichia coli* to freezing damage during the growth cycle. *Proceedings of the Society for Experimental Biology and Medicine* **92**, 499–500.