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The Cryopreservation of *Chlorella*

2. Effect of Growth Temperature on Freezing Tolerance

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Abstract. The temperature at which *Chlorella* 211/8h was grown determined the response to a subsequent stress of freezing to and thawing from − 196° C. Cells cultured at 20° C were the most sensitive to freezing injury; at both higher and lower growth temperatures resistance to damage induced by freezing developed. At all culture temperatures examined the freezing tolerance varied with the age of culture.

Key words: Chlorella – Cryopreservation – Cooling rate – Fatty acid composition – Lipid lateral phase separations.

In a previous paper (Morris, 1976) major differences in the recovery of two strains of *Chlorella* following freezing to and thawing from − 196° C were reported. *Chlorella* 211/7a has a recovery greater than 90%, whilst strain 211/8h had a survival of less than 0.1% at all rates of cooling studied. Glycerol and dimethylsulphoxide were found to be cytotoxic to *Chlorella* 211/8h at concentrations (> 2.5% w/v) useful for cryopreservation.

Since it has been demonstrated that the frost resistance of some freshwater algae (Scholm, 1968), benthic seaweeds (Bird and McLachlan, 1974; Lyutova et al., 1967) and higher plants (reviewed by Levitt, 1972) increase with decreasing environmental temperature, it was decided to determine whether *Chlorella* 211/8h could be made freezing tolerant by a process of cold acclimatization. It has also been reported that the recovery following freezing and thawing of *Escherichia coli* (Toyokawa and Hollander, 1956), *Chlorella* 211/7a (Morris, 1976) and cilliated protozoa (Osborne and Lee, 1975; Simon and Schneller, 1973) varies with the age of culture. Owing to the different growth rates of *Chlorella* at the experimental temperatures studied, it was necessary to determine freezing tolerance on successive days of culture. Finally, the effect of a range of cooling rates to − 196° C on cells grown at different temperatures was examined.

METHODS

Cells. Chlorella fusca var. *vacuolata* Shihira et Krauss, Culture Centre of Algae and Protozoa strain 211/8h was grown in liquid culture as previously described (Morris, 1976).

Freezing and Thawing. Cells from liquid culture were used without further preparation, 1 ml of the cell suspension being added to a 12 × 35 mm sterile polypropylene tube (Sterilin) which was then immersed in liquid nitrogen. From temperature measurements determined as described previously (Morris, 1976) the rate of cooling of the cell suspension from − 5° to − 60° C was found to be 212 ∓ 17° C/min ($N = 54$). Cells were cooled at different rates to − 196° C using the technique previously described (Leibo et al., 1970; Morris and Farrant, 1972). The cells were thawed by rapid agitation of the tube in a water bath at 25° C until the last crystal of ice had melted. The rate of warming from − 60° to − 5° C was 90 ∓ 11° C/min ($N = 20$).

Thermal Shock. 1 ml aliquots of cells in polypropylene tubes were incubated in a water bath at 25° C for 30 min before being placed into a stirred ice bath for 15 min; this produced an exponential but reproducible rate of cooling.

Viability Assay. The assay of viability of the frozen and thawed cells has been described previously (Morris, 1976). The efficiency of plating for unfrozen cells as determined by haemocytometer counts was always greater than 95%. In each experiment there were three replicates for each treatment.

RESULTS AND DISCUSSION

Flasks were innoculated with *Chlorella* 211/8h from the stationary phase of growth (14 days at 25° C). On successive days of culture the cells were cooled to 0° C or − 196° C. There was no loss of viability of *Chlorella* 211/8h upon a reduction in temperature from 25° to 0° C (thermal shock). This is in contrast to reports that cells from the exponential phase of growth

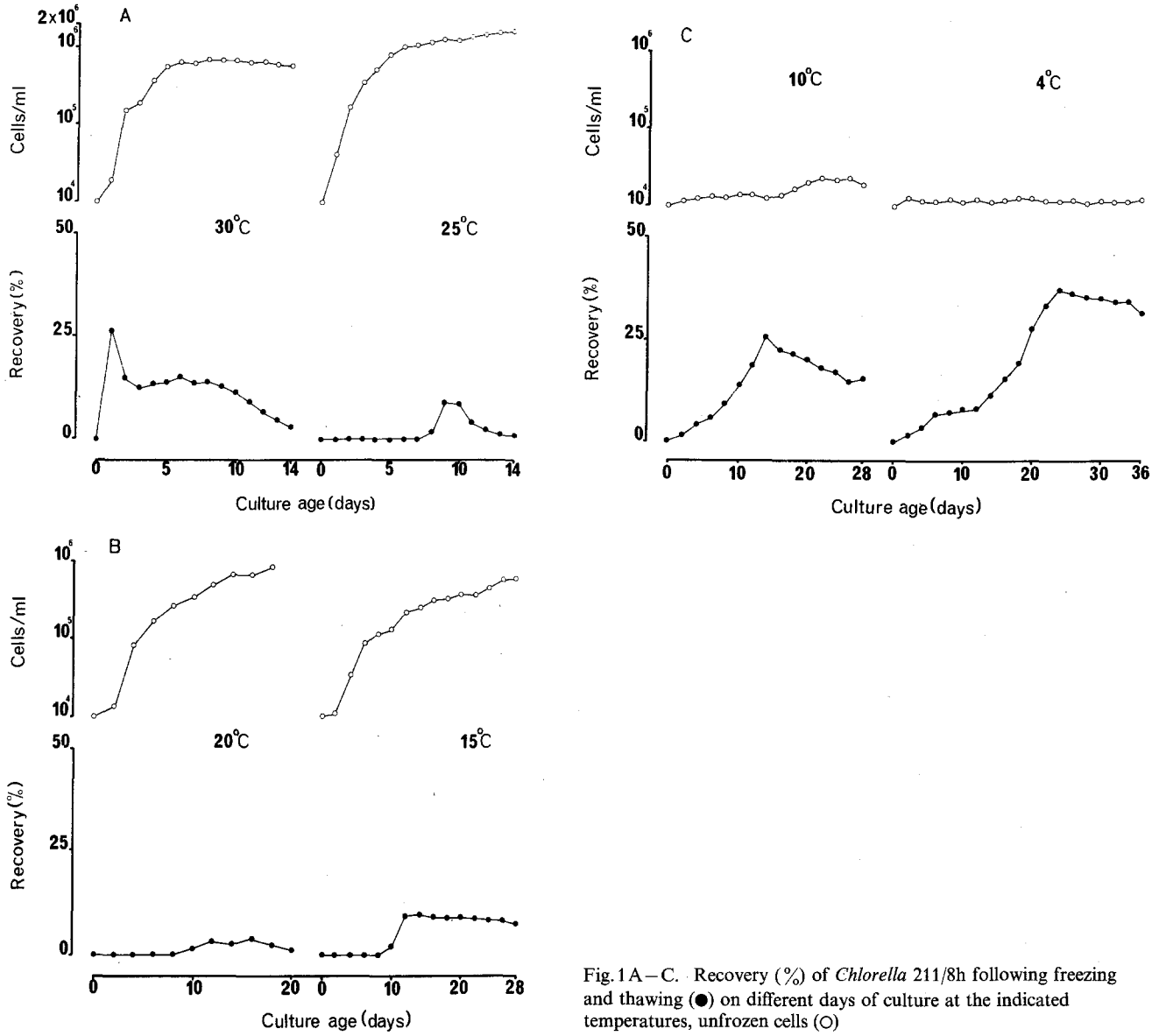

Fig. 1 A–C. Recovery (%) of *Chlorella* 211/8h following freezing and thawing (●) on different days of culture at the indicated temperatures, unfrozen cells (○)

of *Chlorella* 211/7a (Morris, 1976) and synchronous cultures of 211/8b (Lorenzen, 1963; Pirson et al., 1958) are sensitive to damage induced by thermal shock.

With cells grown at 20° C the survival following freezing and thawing was less than 0.1 % on days 0–8 of culture, though there was a small but significant increase in freezing tolerance on days 10–20 (Fig. 1 B). At lower growth temperatures (Fig. 1 B and C) the resistance to injury induced by freezing and thawing increased as the temperature decreased. These results are consistent with the frost tolerance data obtained for algae (Bird and McLachlan, 1974; Lyutova et al., 1967; Scholm, 1968) and higher plants (Levitt, 1972). Increasing the growth temperature to 25° and 30° C (Fig. 1 A) also induced cellular resistance to freezing injury compared with cells grown at 20° C.

At all growth temperatures studied there was a peak in the resistance to freezing damage, and at both older and younger stages of culture there was a reduction in the factor(s) determining freezing tolerance. The age of culture at which this peak occurred increased with decreasing growth temperature, from day 1 at 30° C to 24 days at 4° C. There is thus an inverse correlation between the freezing tolerance acquired. Cells cultured at sub-optimal growth temperatures (30°, 15°, 10° and 4° C) developed the highest degree of freezing tolerance. Whilst cells cultured at or near the optimal temperature for growth (20° and 25° C) were the most sensitive to freezing injury.

Chlorella 211/8h was cultured at 30°, 25°, 15° or 4° C to obtain cells with a maximum freezing tolerance at each of these temperatures. The age of culture

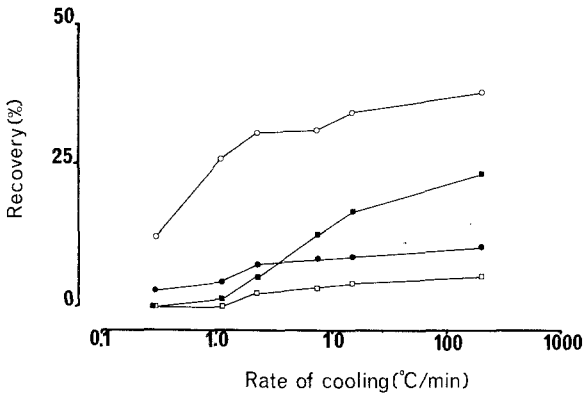

Fig. 2. Recovery (%) of *Chlorella* 211/8h after cooling at different rates to −196° C. Cells were cultured for either 1 day at 30° C (■), 10 days at 25° C (□), 14 days at 15° C (●) or 24 days at 4° C (○)

required to produce cells in this physiological state being determined from Figure 1A–C. The effect of a range of cooling rates to −196° C on these cells was the determined. With all cultures studied the highest recovery following freezing and thawing was obtained at the fastest rate of cooling examined (212° C/min) and fell at slower rates (Fig. 2). In a previous report (Morris, 1976) the recovery of *Chlorella* 211/8h was less than 0.1% at all rates of cooling studied, this being due to the growth temperature (25° C) and the age of culture (7 day) of the cells examined. The recovery of *Chlorella* 211/7a was greater than 90% at all rates of cooling studied.

For most cell-types there is a cooling rate giving optimal survival, with damage increasing at both slower and faster rates of cooling. From these findings it has been argued that two processes of freezing injury occur (reviewed by Mazur, 1970). At cooling rates faster than the optimal, the formation of intracellular ice (brought about by insufficient time for cell water to be lost osmotically) has been implicated as a damaging mechanism. Because of the low value of water permeability of algal cells (Stadelmann, 1966), and the relatively large cell volume (3–15 μm diameter) the probability of intracellular ice formation would be high in *Chlorella* cells cooled at a rate of 212° C/min to −60° C (Mazur, 1963). Intracellular ice formation therefore is not a damaging mechanism of freezing injury for *Chlorella* 211/7a and 211/8h. At rates of cooling slower than the optimal, cells are exposed to hypertonic solutions for longer periods, the time of hypertonic exposure increasing with the decreasing rate of cooling. These hypertonic solutions are either damaging per se (Lovelock, 1953) or induce thermal shock upon a subsequent reduction in temperature (Farrant and Morris, 1973).

It is generally assumed that the cellular membranes are the primary site of freezing and thawing injury. Recently much evidence has been presented that membrane lipids change from a liquid crystalline to a gel structure upon a reduction in temperature (reviewed by Grant, 1975). The extent of these lipid lateral phase separations and the temperature at which they occur are dependent both on the lipid composition and the degree of fatty acid saturation of the membrane (James and Branton, 1971; Shechter et al., 1974). Lipid phase changes have been shown to modify membrane protein topography (James and Branton, 1973; Speth and Wunderlich, 1973; Verkleij et al., 1972) and the response of cells to hypotonic solutions (van Zoelen et al., 1975). Both these factors may be important in determining the response to freezing and thawing. Analyses of the fatty acids and lipids of plants hardened to withstand sub-zero temperatures have shown increases in the unsaturation of fatty acids and the total weight of fatty acids (Gerloff et al., 1966; Kuiper, 1970; Siminovitch et al., 1968). Patterson (1970) has demonstrated that the fatty acids of *Chlorella* 211/8k are maximally saturated at a growth temperature of 20° C, and that at higher and lower growth temperatures an increase in unsaturation occurs. If the effect of temperature on the membrane fatty acids of *Chlorella* 211/8h follows a similar pattern then there is a correlation between the degree of fatty acid saturation and the freezing tolerance. However, as the degree of fatty acid unsaturation increases there is a concomitant increase in the fatty acid chain length, and therefore as the growth temperature is lowered the lipids do not have a lower melting point (Patterson, 1970). If the increase in fatty acid unsaturation is the mechanism by which freezing tolerance is acquired at low growth temperatures it is by some mechanism other than by lowering of the temperature of the lipid phase change. The increase in total membrane lipids observed at extremes of growth temperatures (Patterson, 1970) may be the cryoprotective factor as recently suggested for cells of the black locust tree (Singh et al., 1975). The composition of the membrane lipids of *Chlorella* 211/8k varies with the age of culture (Patterson, 1970) so this may account for the peak values of freezing tolerance at each experimental temperature studied. Further studies using *Chlorella* cells as a model system may thus elucidate some of the factors responsible for frost hardening of plant cells and so provide insights into the mechanisms of freezing injury.

Culturing cells at low temperatures provides a simple and effective method of cryopreservation. Following a 24 day incubation at 4° C the recovery following freezing and thawing was 38%, this is equivalent to results obtained with cells grown at 25° C and frozen in the presence of 5% w/v dimethylsulphoxide (Morris, 1976). As dimethylsulphoxide is cytotoxic to *Chlorella* 211/8h it has to be removed

upon thawing by washing and centrifugation. Cells preserved following growth at low temperatures may be used directly upon thawing. Further work on the application of this technique to other algal cells is now in progress.

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