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Enzymatic Degradation of the Cell Wall of *Chlorella**

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Summary. Up to 80% of the cells of actively growing *Chlorella* species transformed into large, round, osmotically labile cells with cell walls apparently strongly damaged or completely degraded after a 90 h incubation period with a polysaccharide degrading enzyme mixture.

For many years reports have been made on successful experiments to strip vegetative plant cells of their rigid cell wall in order to obtain naked protoplasts which remain alive under favorable conditions. Such cells may regenerate their cell wall and undergo cell division (Takebe *et al.*, 1971; Grambow *et al.*, 1972). Bacterial cells lacking a cell wall became acceptors of viruses which ordinarily cannot adsorb to their cell walls, and the protoplasts of higher plants could be made to unite with one another (Potrykus, 1971).

The most impressive results have been gained using a hybrid which was a cross between *Nicotiana glauca* and *N. langsdorfii*, (Carlson *et al.*, 1972) the morphological, cytological and biochemical characteristics of which correspond to those of the amphiploid hybrid obtained by means of sexuality (Schaeffer and Smith, 1963).

Our interest having been excited by experiments such as those described above, we decided to try to obtain protoplasts from *Chlorella*, although it is known that the cells of at least some *Chlorella* species are protected by a sporopollenin layer (Atkinson *et al.*, 1972), "one of the most extraordinarily resistant materials known in the organic world" (Faegri and Iversen, 1964). This may be the reason why up to now *Chlorella* has withstood the attempts to prepare protoplasts (Atkinson *et al.*, 1972) in spite of the fact that protoplasts of algae were among the first objects studied (Küster, 1939) and that Gabriel 1970 succeeded in preparing protoplasts from *Uronema gigas*, another green alga, and that the harvest and the properties of protoplasts from bacteria (Weibull, 1958), fungi (Ferenczy *et al.*, 1974) and mosses (Binding, 1966) as well as from higher plants (Cocking, 1972) and animals (Harris, 1970) have repeatedly been reported.—As long as it is not proven, that the cell-wall damaged cells we prepared from *Chlorella* are actually naked protoplasts, we shall call them spheroplasts.

* Dedicated to Professor Dr. W. Menke on his 65th birthday.

Materials and Methods

The *Chlorella* strains *Chl. saccharophila* 211-9a and *Chl. ellipsoidea* 211-1b, which we used originally came from the algae collection in Göttingen (Koch, 1964). They were cultivated in a mineral nutrient medium according to Ruppel (1962). As osmotic stabilisers, 0.35 M sorbitol and 0.35 M manitol were added. On the basis of previous experiments, the following mixture of enzymes in 10 ml medium was used: 2% cellulase Onozuka SS, 1% hemicellulase Roth 2–6215, 2% pectinase Roth 2-6839, with 2% bovine serum albumen added. The pH-value was adjusted to 5.5 with KOH or HCl as required. During incubation the samples are lightly shaken at a temperature of 25°–27° C (minimum 2 h, maximum 24 h). All the work was carried out under sterile conditions. The solutions including enzymes and osmotica were pressed through a Millipore filter SA 78 BUC with a pore diameter of 0.8 μm after the addition of penicillin-G-potassium salt and Orthocid (50 mg/l). The addition of the fungicides proved useful, as apparently one or more of the enzyme preparations contained fungus spores. The liquid cultures had air bubbled through them and were illuminated from the two sides with an intensity of 7000–10000 Lux.

Results

The cells of *Chl. saccharophila* are relatively large. Cells containing autospores have an average size of 7.3 μm, the autospores a size of 3.7 μm (cell size = smallest diameter of the cell). The cells are ellipsoid. Of the *Chlorella* species studied by Soeder, 1963, they have the largest axis ratio: the autospores 1.45, the sporangia 1.8. Therefore the two to three times larger and round spheroplasts were easily distinguished from unaffected cells under the microscope.

In order to produce spheroplasts an unexpectedly long incubation time was necessary. Although the cells in the medium reproduced themselves six times within 24 h, incubation times with the enzymes up to 24 h went without any appreciable effect. Only after a longer incubation period in the air-bubbled cultures did cells with modified cell walls appear. The fact that *Chlorella*'s cell wall was either strongly damaged by this method or completely destroyed was seen by the modified form and size as well as by an increased osmotic lability. When spheroplasts were transferred from the enzyme solution into aqua dest. they burst after a few minutes. Another criterion which showed that we had cell wall damaged cells, was their sensitivity to mechanical pressure. They burst directly when a cover slip was lightly pressed against the slide.

Fig. 1 shows spheroplasts, in which approximately 80% of the cells from an air-bubbled culture of *Chl. saccharophila* were transformed after a 90 h incubation with enzymes. Unchanged cells included in the figure, make clear the difference between spheroplasts and normal cells. The entire length of the elliptical cells is still smaller than the diameter of the round spheroplasts. An adjustment of the Onozuka-cellulase concentration to 1% produced only a few spheroplasts after 90 h, a higher concentration than 2% produced no increase in the percentage of spheroplasts. An experiment with incubation at a higher temperature (32° C), in order to raise the cellulase activity, was unsuccessful: within the time period observed no spheroplasts appeared. Another experiment with the incubation of *Chlorella* cells in the dark without air-bubbling also gave negative results. Some orientating experiments with *Chl. ellipsoidea* had essentially the same results. In this case, however, no more than 20% of the cells showed the form characteristic of spheroplasts.

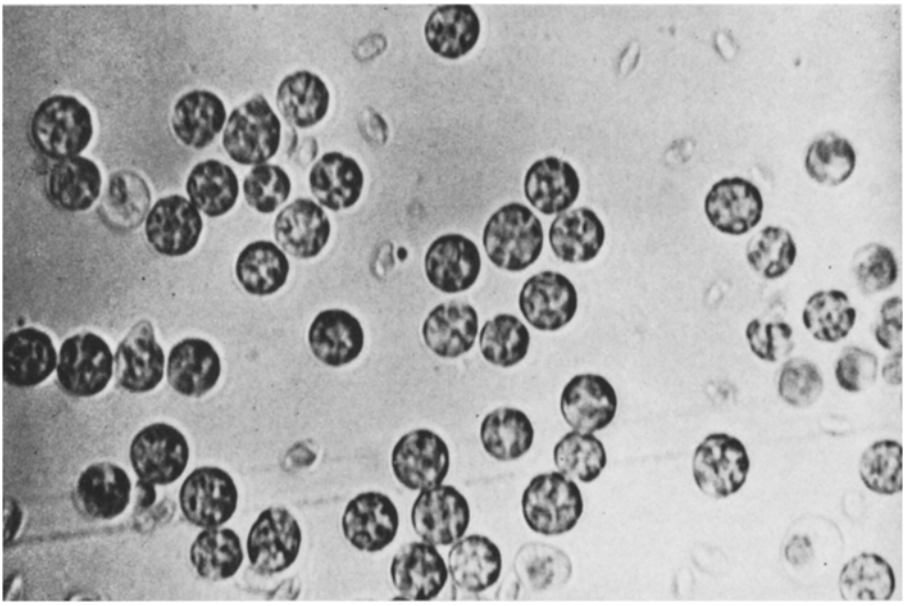

Fig. 1. *Chlorella saccharophila* after 90 h incubation in Ruppels mineral solution plus the following additions: 0.35 M sorbitol, 0.36 M manitol, 2% cellulase Onozuka SS, 1% hemicellulase (Roth), 2% pectinase (Roth), 2% bovine serum albumen. Temp. 25–27° C. continuous light, approximately 80% spheroplasts. Magnification. × 800

Discussion

For the experiments described *Chlorella saccharophila* 211–9a was chosen because its cell wall is probably not protected by an outer layer of sporopollenin. In spite of this, however, the cells proved quite stable against attack by cellulases. According to Cocking (1972) the cellulase Onozuka, which makes up the main part of the enzyme combination used here, contains cellulase C_1 (attacking native and crystalline cellulose), cellulase C_x (attacking amorphous cellulose), cellobiase, xylanase, glucanase, pectinase, lipase, phospholipase, nucleases, and other enzyme activities. Although the enzyme combination in our experiments was extended by the addition of further polysaccharide-degrading enzymes, an enzyme capable of degrading the main constitutent of the outer layer of the cell wall seems not to have been present in sufficient quantities, as shown by the delayed degradation of the cell wall.

This leads one to believe the enzyme's effect might be stronger on cells during certain early stages in the developmental cycle. Efforts to synchronize cell division in *Chl. saccharophila* gave no satisfying results in spite of the fact that we did not add sucrose or other organic material to the mineral solution.—We also tried incubation experiments with *Chl. ellipsoidea* 211–1b, which, although they gave a smaller yield of spheroplasts, show that it is not impossible to produce spheroplasts from other *Chlorella* species whose cell wall presumably presents less favorable conditions for the degradation by enzymes. Some observed couples (Fig. 2) of spheroplasts, which showed no separating membrane at their point of

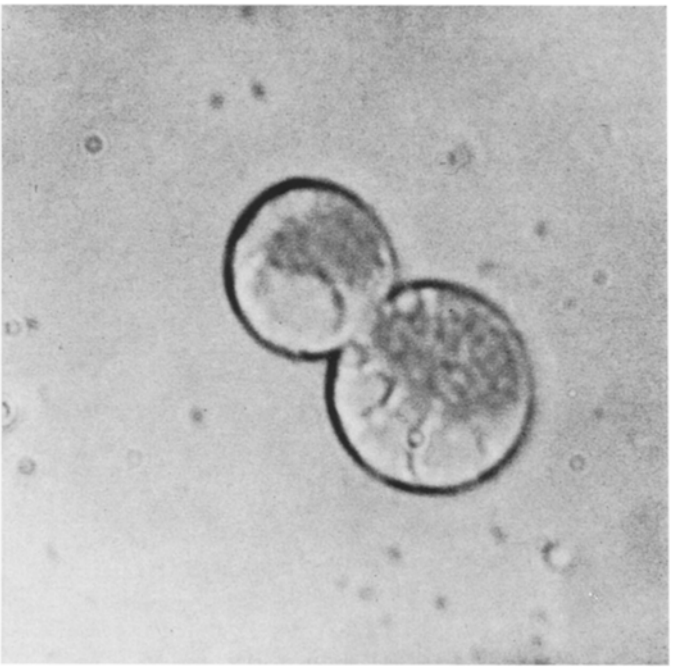

Fig. 2. *Chlorella saccharophila*. Two spheroplasts lying in close contact. Conditions as in Fig. 1. Magnification ×1350

attachment despite repeated focussing of the microscope, led us to suggest the possibility of cell fusions. Conclusive proof for such a conjecture is still lacking at the present.

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