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Effect of Increasing Copper and Salinity on Glycerol Production by *Dunaliella salina*

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Dunaliella salina is a green marine flagellate whose main storage product is glycerol. Its ability to grow in waters which vary greatly in salinity is due to its ability to form glycerol, the intracellular concentration of which is directly proportional to the extracellular salinity (Borowitzka & Brown 1974; Ben-Amotz & Avron 1978).

Copper is an essential trace element needed in small quantities by algae for plastocyanin production. However, at high concentrations it acts as an effective algicide. Lethality due to copper is greatest with high activity of free cupric ions (Sunda & Guillard 1976). The cupric ion activity is related not only to concentration of copper but also to pH and concentration of chelators (Hawkins & Griffiths 1982). Dunaliella species have been shown to have an unusual resistance to copper toxicity (Wikfors & Ukeles 1982; Lustigman et al.1985).When copper concentration is not lethal, it inhibits reproduction resulting in an increase in the pigment concentration (Lustigman 1986). It also delays log phase of growth, slowing the rate of increased cell population density (Pace et al. 1977).

We examined the effects of lethal and sublethal concentrations of copper on growth and glycerol production in Dunaliella salina, in order to determine if adaptation to copper toxicity will result in an increase in production of glycerol.

MATERIALS AND METHODS

The culture of Dunaliella salina was isolated from a Long Island, N.Y. salt marsh. It was grown on 0.2μ millipore filtered modified DC medium of 32‰S, pH 7.8 (Provasoli et al. 1957). This is considered to be isotonic to sea water. The cultures were tested periodically by plating on bacteriological media and were found to be free of bacterial contamination. Transfers were also made to hypertonic medium (6.0‰S). Test tubes and other glassware were acid washed, sterilized, filled with 15ml of medium and incubated 24 hours prior to inoculation. Cell counts were made to establish an inoculum of approximately 10^6 cells. Cultures were maintained in an incubator at 22-25°C, continuously illuminated with 2000 lux from GE cool white 30 watt fluorescent bulbs. Cultures were maintained for 28 days in order to determine the growth curve.

Copper was supplied as $CuSO_4 \cdot 5H_2O$ from 1% Cu^{++} stock solution to achieve

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concentrations of 0,5,10,20,30,40,50 ppm Cu^{++}. These values were tested by atomic absorption analysis of uninoculated samples and found to be accurate. In addition, the Cu^{++} concentrations of the cells and the medium at the end of the experimental period were measured. A total of 18 cultures were tested for each experimental value.The concentration and solubility of Cu^{++} are determined by pH and chelation. In addition to buffering pH, TRIS (tris hydroxy-methyl-aminomethane) chelates copper. It regulates Cu^{++} activity so that it remains constant throughout the course of the experiment.

At 7, 14, 21 and 28 days samples were removed from the cultures and counted using a Neubauer hemocytometer. At 21 days, 8 ml samples containing approximately 4×10^6 cells/ml were removed from the cultures .The samples were then centrifuged at 1500 Xg for 10 minutes. Pellets were washed twice in 5mM PO_3 buffer, pH 7.8, with NaCl concentration in accordance with the medium in which a given sample was grown. Extraction and analysis for glycerol was done following an adaptation of the methods devised by Ben-Amotz & Avron (1973).The washed algal pellet was resuspended to a final volume of 1 ml in 15 mM PO_3 buffer, pH 7.5, which also contained NaCl as above. The protein and pigments were precipitated by the addition of 0.1 ml of 30% trichloroacetic acid (TCA) to each tube and thoroughly mixed. A clear supernatant was obtained by centrifugation at 1500 Xg for 10 minutes at 0^0 C . A 0.5ml quantity of protein-free supernatant was then pipetted into new test tubes for the glycerol assay. In addition to the unknowns, glycerol standards were prepared. To all these tubes, 0.1 ml of 10N H_2SO_4 was added, followed by 0.5 ml of 0.1M sodium periodate. After 5 minutes, 0.4 ml 1M sodium arsenite was added. After about 5 minutes, when the color had faded, 0.5 ml was transferred from each tube to a fresh test tube. To this aliquot, 5 ml of chromotropic reagent solution was added and the tubes boiled for 30 minutes. When the tubes had cooled, they were read at 570 nm on a Beckman DB-G spectrophotometer.

RESULTS AND DISCUSSION

The reproduction rate of the algae decreased in hypertonic medium so that the cell densities at hypertonic, 60‰S, were less than at isotonic, 32‰S (Tables I and 2). Since copper sulfate is an algicide, its major effect is to retard the growth of algae. Cultures of Dunaliella salina showed a decrease in cell density starting with 5 ppm Cu^{++}. After a marked decrease in population with concentrations up to 30 ppm Cu^{++}, there was a leveling off of the effect. We found that 50 ppm Cu^{++} was the maximum copper concentration that could be tolerated by our Dunaliella salina cultures. As salinity increased, so did resistance to the lethal effects of copper. Cultures raised at 60‰S did not display as marked a per cent decrease in density as those grown at 32‰S. Since the cultures were maintained for 28 days, the conditions were such as to select for those individuals that were resistant to Cu^{++}. By 21 days, the cultures show an increase in population density as these cells reproduce. Cultures exposed to copper show a delayed log phase of growth and never reach the same cell densities as control cultures.

The most statistically significant effect on glycerol per cell was its increase with increasing salinity (Table 3). Concomitantly, there was a smaller, but still statistically significant increase due to copper concentration with the result that the addition of copper to cultures produced an increase in the amount of glycerol per cell at both salinities employed. Since sublethal concentrations of copper delay log phase of growth, the resulting

Table 1. Number of cells (10^6) /ml at 32‰S with increasing copper concentration

Cu^{++}(ppm)	7	14	21	28 days
0	1.82±0.24	3.15±0.60	4.27±0.47	4.35±0.56
5	1.10±0.33	3.09±0.51	4.16±0.59	4.09±0.34
10	0.78±0.19	2.20±0.45	3.53±0.83	3.64±0.42
20	0.63±0.21	1.30±0.20	3.01±0.27	3.12±0.55
30	0.51±0.14	1.35±0.45	2.04±0.68	2.35±0.22
40	0.47±0.16	1.31±0.25	2.23±0.69	2.41±0.38
50	0.39±0.10	1.40±0.60	2.17±0.78	2.26±0.19

Table 2. Number of cells (10^6)/ml at 60‰S with increasing copper concentration

Cu^{++}(ppm)	7	14	21	28 days
0	1.43±0.57	2.16±0.62	2.88±0.31	3.00±0.55
5	1.01±0.28	1.44±0.32	2.01±0.75	2.34±0.62
10	0.82±0.43	1.47±0.54	1.90±0.56	2.17±0.44
20	0.64±0.31	1.49±0.33	2.05±0.59	2.25±0.31
30	0.46±0.22	1.39±0.23	1.60±0.63	2.04±0.47
40	0.38±0.17	1.33±0.10	1.76±0.55	1.93±0.25
50	0.32±0.08	1.31±0.16	1.63±0.22	1.81±0.55

Table 3. Concentration of glycerol/cell (gm X 10^{-10}) at 21 days

Cu^{++}(ppm)	32‰S	60‰S
0	5.77±0.59	7.52±0.78
5	5.98±0.93	8.45±0.84
10	5.27±0.30	9.11±0.55
20	6.85±0.21	9.27±0.69
30	7.32±0.34	10.32±0.66
40	7.94±0.43	10.60±0.50
50	8.46±0.45	11.03±0.42

cells have a higher concentration of the storage product, glycerol. According to statistical analysis, salinity and copper did not interact significantly.

However, while glycerol concentration per cell increased, cell density decreased so that total yield of glycerol was not greater with copper treatment than in the non-treated controls.

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