BRIEF NOTE

NUTRIENT CONCENTRATION REQUIREMENTS FOR *CHLORELLA SOROKINIANA*[1]

CLYDE EYSTER, Mobile College, Mobile, AL 36613

OHIO J. SCI. 78(2): 79, 1978

Minimum, optimal and maximal concentration of essential nutrient elements for the growth of *Chlorella sorokiniana* was determined. Growth measurements included optical density and packed cell volumes.

Culturing apparatus: A photothermostat of the type described by Myers (1950) was constructed. The interior dimensions were 22 inches long by 3 inches wide by 7½ inches deep and the plastic Lucite aquarium lid accommodated 22 test tubes held snugly in place.

Water at 38.8° C was continuously circulated by a ⅓ hp electric pump. A filter inserted between the pump and the aquarium kept the water clear. The temperature controller was adjusted so that a temperature of 39° C was maintained in the aquarium bath and the average operating temperature was found to be 38.8° C, with a standard deviation of ±0.16° C.

The culturing apparatus was equipped with 2 banks of lights, a bank for each side of the aquarium bath. Each bank has three 48-inch Sylvania cool white power tubes, providing the tube cultures a light intensity of 925 ft-candles from each side.

Agitation and carbon dioxide for the test tube cultures was provided by passing into each tube culture a mixture of 5% CO_2-Air through a glass bubbling tube. Each bubbling tube was 20 cm long and was constricted approximately 3 cm below its upper end in order to hold a small cotton plug. The bubbling tube was fitted into the top of the test tube by means of a cotton plug wrapped with gauze. A paper collar 5 cm in length was made to extend above the cotton plug to protect the culture from microbial contamination, when the bubbling tube was elevated for optical density measurements.

Two manifolds, each providing 12 outlets, were constructed from brass tubing and needle valves. Autoclaved surgical rubber tubing connected the needle valve outlets with the bubbling tube of each test tube culture. The 5%CO_2 - Air, was humidified by passing it through a sterilized 300 ml side-arm bubbling flask containing 100 ml of distilled water, and then directed into the manifolds which were cleaned and steamed before being installed.

Culture procedure: Cultures were grown in triplicate in modified Knop's medium (Eyster 1966; 1967), which was modified to contain urea, micro-nutrient trace elements, and ethylene diamine tetraacetate (EDTA) as a chelating agent. Modifications of Knop's medium for determining elemental nutrient requirements are shown in table 1.

It was found necessary to use triple

[1]Manuscript received January 19, 1977 and in revised form as a note May 19, 1977 (#77-8).

TABLE 1

Knop's Medium modifications for determining the elemental nutrient requirements of Chlorella sorokiniana.

Element*	Deleted	Substituted	Concentration Regulator
Magnesium	Magnesium sulfate	Sodium sulfate	Magnesium sulfate
Sulfur**	Magnesium sulfate	Magnesium chloride	Sodium sulfate
Potassium***	Potassium dihydrogen phosphate	Sodium dihydrogen phosphate	Potassium chloride
Phosphorus	Potassium dihydrogen phosphate	Potassium chloride	Sodium dihydrogen phosphate
Nitrogen	Urea, nitrate†	—	—
Iron	Ferric nitrate	—	Ferric nitrate
Calcium	Calcium chloride	—	Calcium chloride
Zinc	TEM•	TEM minus Zinc chloride	Zinc chloride
Manganese	TEM°	TEM minus Manganese sulfate	Manganese sulfate
Copper	TEM*	TEM minus Copper sulfate	Copper sulfate

*Amount contributed by inoculum was included in calculating element levels.
**Trace elements ordinarily supplied as sulfates were supplied as chlorides.
***pH was adjusted to 6.8 with NaOH instead of KOH. The disodium salt of EDTA was used instead of its potassium salt.
†Or other nitrogen sources.
•Trace element mixture: manganese, copper, molybdenum, cobalt salts and zinc chloride.
°Trace element mixture: zinc, copper, molybdenum, cobalt salts and manganese sulfate.
*Trace element mixture: manganese, zinc, molybdenum, cobalt salts and copper sulfate.

glass distilled water because the single distilled water had about 50 ppb copper ions. Analyses for copper by the use of zinc dibenzyldithiocarbamate in CCl_4 (Stone *et al* 1953) revealed that KH_2PO_4 had appreciable (88 mg/mole) copper as a contaminant, and that $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and H_3BO_3 had smaller amounts (42 mg/mole, 56 mg/mole, and 2 mg/mole, respectively). KNO_3 was found to have only 0.8 mg copper per mole compared with 4800 mg per equivalent of urea. This perhaps explains why Walker (1953) could demonstrate a copper requirement with nitrate in the medium but not with urea in the medium. Copper deficiency symptoms are more pronounced at relatively high levels of EDTA. It is recommended that copper essentiality efforts include, first, the purification of water, KH_2PO_4, $MgSO_4$, $CaCl_2$, urea, and H_3BO_3 for copper removal, and second, an increase in concentration levels of EDTA.

For my studies, 4.5 ml of culture medium were placed in each test tube. A glass bubbling tube or "stem", fitted with a 5 cm long paper collar and a cotton plug, was inserted into each test tube, which was then steam-sterilized in an autoclave at 10 lb pressure for 10 minutes. Sterile urea (0.25 ml) and bacteria-free inoculum (0.25 ml) were then added to each cooled tube culture by means of a heat-sterilized 1.0 ml B-D tuberculin syringe fitted with a long sterile needle. This needle was inserted through the hollow center of the stem to deliver, in 2 operations, the urea and the inoculum. It was customary to prepare the medium so that 4.5 ml contained the nutrients intended for 5.0 ml. This technique allowed the addition of 0.25 ml of subcultured inoculum and 0.25 ml of sterile urea solution (Eyster 1966; 1967).

A bacteria filter apparatus with a fine fritted disc was used to produce bacteria-free solutions of urea. An electric instrument sterilizer was utilized to heat-sterilize the syringes and needles. Tubes of trypticase soy broth and tubes of nutrient agar were used for the purpose of checking the sterility of the *C. sorokiniana* cultures periodically.

Experimental measurements: Growth at the end of 23 hours was measured by obtaining absorbance readings at 550 mμ. Optical absorption by *C. sorokiniana* cells

TABLE 2

Summary of mineral nutrient requirements for the growth of Chlorella sorokiniana.

Nutrient Element	Minimum Concentration	Optimum Concentration	Maximum Concentration
Nitrogen	1×10^{-3}M	1×10^{-2}M to 5×10^{-2}M	2×10^{-1}M
Magnesium	1×10^{-5}M	1×10^{-4}M to 4×10^{-2}M	1×10^{-1}M
Sulfur	1×10^{-5}M	2×10^{-4}M to 5×10^{-2}M	1×10^{-1}M
Potassium	1×10^{-5}M	5×10^{-4}M to 1×10^{-1}M	5×10^{-1}M
Phosphorus	5×10^{-5}M	5×10^{-4}M to 2×10^{-2}M	1×10^{-1}M
Calcium	1×10^{-5}M	5×10^{-4}M to 3×10^{-3}M	1×10^{-2}M
Iron	1×10^{-6}M	2×10^{-5}M to 1×10^{-3}M	2×10^{-3}M
Zinc	1×10^{-6}M	1×10^{-5}M to 1×10^{-2}M	—
Manganese	3×10^{-7}M	1×10^{-5}M to 1×10^{-2}M	—
Copper	10^{-12}M	10^{-8}M to 10^{-3}M	2×10^{-3}M
EDTA	zero	3×10^{-4}M to 2.5×10^{-3}M	4.5×10^{-3}M

did not follow Beer's Law at densities above 0.850 and direct absorption readings of dense cultures were less than the calculated total absorption values derived from the absorption of diluted aliquots. It was our practice to place 0.50 ml of dense culture in 9.50 ml of fresh medium, read the absorbance of the mixture, and then multiply the absorbance value by 20, giving a total absorbance value for the dense culture. The most dense cultures had to be diluted 40-fold. Additional measurements included pH and packed cell volume determinations (Eyster 1966; 1967).

A summary of the elemental nutrient requirements for the growth of *C. sorokiniana* is given in table 2, which is based on the data reported by Eyster (1966; 1967), and in a specially prepared manuscript (Eyster 1976) with complete supportive data. Nutrient elements which were found to be required were N, P, Mg, S, K, Fe, Ca, Mn, Zn, and Cu. On the contrary, there was no indication that B, Mo, Na, Co, Sr, and Cl were required.

Acknowledgments. The research reported in this paper was sponsored by the Environmental Sciences Division, USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, United States Air Force, Brooks AFB, Texas, under Contract No. AF 41 (609)-2414 with Dayton Laboratory of Monsanto Research Corporation.

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