Polysaccharide Produced by *Anacystis nidulans*: Its Ecological Implication

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Received for publication 23 June 1972

An extracellular polysaccharide from *Anacystis nidulans* was extracted from cell-free medium. Analysis showed that the polysaccharide consisted of glucose, galactose, and mannose in the ratio of 60:14:20. The production of polysaccharide depends on the age of culture, the growth temperature, and the form of nitrogen available.

Several freshwater green and blue-green algae are known to excrete varying amounts of organic compounds into their surrounding environments. Polypeptide and free amino acid liberation by a species of *Anabaena* has been reported by Fogg (9). Organic acid production from species of *Chlamydomonas* has been studied by Allen (2). Extracellular carbohydrate polymer production has been reported for *Anabaena cylindrica*, *Nostoc muscorum*, and *Palmella mucosa* (4, 5, 12). Almost all of the common monosaccharide units have been identified in these polymers. Type and amount of polysaccharide production depend on the species employed, and quantitative data are available from papers of Lewin (11) and Moore and Tischer (13). Species of green algae studied by Lewin (11) produced extracellular polymer ranging from 3 to 113 mg per liter, whereas mucilaginous species analyzed by Moore and Tischer (13) produced the polymers in concentrations from 174 to 557 mg per liter of growth medium.

This study was undertaken to determine the extent and composition of polysaccharide production by the blue-green alga *Anacystis nidulans*. Some of the nutritional factors which might influence the polymer production were also examined because of their potential significance during algal blooms in Lake Erie.

**MATERIALS AND METHODS**

**Culture and media.** A bacteria-free, unialgal isolate of *A. nidulans* was cultured in the medium "c" of Kratz and Myers (10) composed of: MgSO₄·7H₂O, 0.25 g; K₂HPO₄, 1 g; Ca(NO₃)₂·4H₂O, 0.025 g; KNO₃, 1 g; Na₂H₃C₆O₆·2H₂O, 0.165 g; Fe₂(SO₄)₃·6H₂O, 0.004 g; and distilled water to make 1 liter (final pH 7.5). Cultures were grown in 3-liter Fernbach flasks in a Psychrotherm shaker incubator (100 rev/min) at 25 or 40 °C under the continuous illumination of 130 foot candles from a bank of cool, white fluorescent tubes and with 5% CO₂ in air added to the incubator.

**Isolation of the polysaccharide material and measurement of cell dry weight.** Samples were withdrawn aseptically at 2-day intervals. Cells were removed by centrifugation, and total soluble polysaccharide in the supernatant fluid was measured by using the anthrone procedure as described by Ashwell (3). Dry weights were determined after drying the cells in tared aluminum cups overnight at 100 °C. At the end of the growth period, the cells were removed by centrifugation, and the cell-free extracts were concentrated to one-tenth volume in a rotatory evaporator at 60 °C. After dialysis, the extracellular polysaccharides were precipitated by adding three volumes of cold 95% ethanol. The precipitate was collected by centrifugation, washed with absolute ethanol, dried, and weighed. The harvested cells were killed with 0.5 ml of a 2:1:1 (v/v/v) mixture of chlorobutane, chlorobenzene, and dichloroethane (11), and capsular polysaccharides were extracted with 50 ml of distilled water at 5 °C for 24 hr, precipitated with three volumes of ethanol, and weighed. To extract water-soluble intracellular polysaccharides, the cells were ruptured by ultrasound (Bronson sonifier, model S110) for 30 min. The supernatant fluid was treated with 10% trichloroacetic acid to precipitate protein, and the polysaccharides were precipitated with ethanol from the protein-free solution.

Polysaccharide material was also extracted by the alkali extraction method of Bishop et al. (4). The culture of *Anacystis* was made alkaline by adding sodium hydroxide (4:1, w/v). The mixture was boiled for 6 hr under reflux, filtered through sintered glass, adjusted to pH 4 with HCl, and dialyzed for 3 days at 4 °C. The nondialyzable material was isolated by freeze-drying.

**Hydrolysis and chromatography.** Polysaccharide samples (50 mg) were hydrolyzed in 5 ml of 0.7% H₂SO₄ in sealed ampoules for 6 hr at 100 °C. Barium carbonate was added to neutralize the excess acid and BaSO₄ was removed by filtration. Supernatant fluids
were concentrated under reduced pressure at 60°C, and monosaccharides were separated by descending paper chromatography on Whatman no. 1 sheets with three different solvent systems: butanol-acetic acid-water (4:1:5, v/v/v), ethyl acetate-pyridine-water (8:2:1, v/v/v), and isopropanol-water (4:1, v/v). Sugar spots were located on the dried, developed chromatograms by a dip in alkaline silver nitrate (14). Known sugars were included with each run.

Molar ratios of the monosaccharides were determined by gas-liquid chromatography of the hydrolyzates by the procedure of Albersheim et al. (1). The polysaccharide material (20 mg) was hydrolyzed with 2 N trifluoroacetic acid for 2 hr at 121°C. After evaporating the acid, the hydrolysate was reduced with sodium borohydride in 1 N ammonia and acetylated with acetic anhydride, and a 10-μl amount was injected into a Varian model 200 gas-liquid chromatography apparatus.

To determine the effect of nitrogen sources, the algae was grown on various nitrogen sources supplied at a level of 1 mg of culture medium per ml. The response was measured both as dry weight of cells and as extracellular polysaccharide production on the dialyzed cell-free supernatant fluids.

RESULTS AND DISCUSSION

Analysis of spent growth medium and capsular and alkali-extracted polysaccharides showed the presence of glucose, galactose, and mannose as components. The average molar ratio for glucose-galactose-mannose was 66:14:20. Chromatographic and colorimetric analysis did not reveal hexuronic acids or ninhydrin-positive components. The composition of polysaccharide was similar to the sugar moiety of the cell wall of *Anacystis* as reported by Drews and Gollwitzer (7), although these authors also tentatively identified fucose as a cell wall constituent. No fucose could be identified in the present study.

The amount of extracellular polysaccharide, determined gravimetrically after ethanol precipitation, was 306 mg per liter of growth medium after 21 days at 25°C. Capsular and water-soluble intracellular polysaccharide accounted for 10 and 15 mg per liter, respectively. Subsequent experiments gave an average of 414 mg (range 360 to 446 mg) of extracellular polysaccharide per liter and 12.5 mg (range 8 to 16 mg) of capsular polysaccharide per liter. Intracellular polysaccharide was not determined. The extensive accumulation of extracellular polysaccharide as compared with capsular polysaccharide may be attributed to agitation during growth and has been reported with *Anabaena flos-aquae* (13). After extraction and removal of ethanol-soluble as well as ethanol-precipitable material, 0.0826 g of cell residue per liter of medium remained.

Production of extracellular polysaccharide, as determined with the anthrone reagent, in comparison with cell growth at 25 and 40°C over a period of 14 days is presented in Table 1. The total anthrone-positive sugar values are considerably less than expected on the basis of the gravimetric determination of total alcohol-precipitable polysaccharide. Although incubation time (21 versus 14 days) might account for some of the difference, it is assumed that some polysaccharide is lost during our analytical procedure. A comparison of the values shown in Table 1 indicates that cells grow much more rapidly at 40°C than at 25°C. Total cell growth is nearly the same after 12 days at both temperatures and could be attributable to depletion of a limiting nutrient in the growth medium. Production of total extracellular polysaccharide during the growth period was higher at 25°C than at 40°C. Also, the calculated rate of polysaccharide production per unit of cell synthesis ranged from two- to threefold greater at 25°C than at 40°C. It appears that CO₂ is fixed and directed toward polysaccharide synthesis preferentially at the lower temperature. The 14-day values may be misleading because the low total cell weight at 40°C probably reflects cell lysis, and this would bias the calculated amount of polysaccharide produced per gram of cells.

*Anacystis* is one of the blue-green algae responsible for algal blooms in fresh water (8), and most fresh water bodies have a temperature lower than 30°C. It is therefore likely that soluble polysaccharides are produced which diffuse into the water and cause a greater organic enrichment than can be accounted for by many procedures used to measure primary productivity. The report of Moore and Tischer

| Time (days) | Cell dry wt (μg/ml) | Extracellular polysaccharide (μg/ml) | Calculated polysaccharide (mg/g of cell) |
|-------------|---------------------|--------------------------------------|----------------------------------------|
| 2           | 60                  | 258                                  | 250                                    |
| 4           | 112                 | 293                                  | 241                                    |
| 6           | 163                 | 324                                  | 288                                    |
| 8           | 292                 | 384                                  | 188                                    |
| 10          | 439                 | 396                                  | 134                                    |
| 12          | 455                 | 493                                  | 160                                    |
| 14          | 500                 | 325                                  | 128                                    |
| Control     | 0                   | 0                                    | 0                                      |

* * Anthrone equivalent.
(13) on polysaccharide production by several green and blue-green algae in life support systems also supports the present conclusion. The significance of bacterial extracellular polysaccharides in aquatic ecosystems has been reported (8), and the algal polysaccharides undoubtedly have a similar influence.

The influence of several sources of nitrogen on cell yield and polysaccharide production after 10 days at 25°C is presented in Table 2. Although all media contained less available nitrogen, and therefore supported less cell growth, than the Kratz medium used to produce the data in Table 1, relative influences can be compared. Nitrate supported more growth and polysaccharide production than either ammonium or urea, which indicates that nitrate is more metabolizable than ammonium or urea as a nitrogen source. However, the amount of polysaccharide produced per gram of cells is significantly higher in the presence of the less metabolizable ammonium or urea nitrogen than in the presence of nitrate. This suggests that the available carbon source (CO₂) is directed toward extracellular polysaccharide production rather than toward cell material when either total available nitrogen is a limiting nutrient or when ammonium or urea are the nitrogen sources. Therefore, nitrate concentration has more relevance than total available nitrogen in determining the fate of metabolically fixed CO₂. As an ecological consideration, we would predict rapid growth of Anacystis nidulans (potential algal bloom) only after the process of nitrification has converted reduced nitrogen (e.g., from organic pollutants) to nitrate.

**ACKNOWLEDGMENTS**

This study was supported by research grant no. A-024-OHIO from the Office of Water Resources Research, U.S. Department of Interior. Cultures of A. nidulans were kindly provided by Jack Myers.

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