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Chelating Properties of Extracellular Polysaccharides from *Chlorella* spp.

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***Chlorella stigmatophora* LB 993 was grown in artificial seawater under controlled conditions. The production of cell wall polysaccharides attached to the cells and dissolved in the growth medium was monitored during algal growth. Preliminary characterization of the dissolved polysaccharides of *C. stigmatophora* and other *Chlorella* species is presented. The capacity of dissolved polysaccharides of *C. stigmatophora* to bind toxic heavy metals was also studied and compared with that of polysaccharides produced by other marine *Chlorella* species. The differences in metal-complexing capacity observed for dissolved polysaccharides obtained from various *Chlorella* species is attributable to differences in the composition of the polysaccharides, notably the uronic acids content.**

Production of extracellular polysaccharides has been detected in several classes of algae. Most of the research in this area has been done on marine (9) and soil algae (18). The cells of certain green algae characteristically form a mucilaginous capsule composed of polysaccharides (16). When unicellular green algae grow in batch cultures, polysaccharide production is more pronounced during the stationary phase of growth. In *Chlamydomonas mexicana* growing in liquid medium, polysaccharide production was dependent upon growth status, with more polysaccharides being produced after the cessation of cell division (14). However, significant release of polysaccharides into the medium during the logarithmic phase of growth has also been reported (11, 16).

Among the functions attributed to extracellular polysaccharides, it was suggested that they act as natural metal chelators and thus reduce metal toxicity (4, 15, 23). The effect of complexation of divalent cations by natural organic ligands excreted by various algae is well documented (3, 12, 15, 21). However, very little is known of the chemical nature of those organic ligands.

The purpose of the present study is to monitor cell wall polysaccharide production in *Chlorella stigmatophora* LB 993 and to provide preliminary characterization of its chemical nature with regard to toxic heavy-metal-complexing capacity compared with that of polysaccharides of other *Chlorella* species.

MATERIALS AND METHODS

Algal culture. *C. stigmatophora* LB 993, *Chlorella luteoviridis* 211-5a, and *Chlorella salina* 211-25 were grown in batch culture by using artificial seawater prepared according to the method of Jones et al. (13), omitting EDTA. The cells were grown in 1-liter columns, 6 cm in diameter, at 24 ± 1°C. The cultures were maintained under continuous illumination with fluorescent Westinghouse "cool-white" light at 150 $\mu E\ m^{-2}\ s^{-1}$. The medium was aerated with sterile air containing 2 to 3% CO_2. Growth was monitored by measuring cell counts in a hemacytometer and by measuring dry matter. For the dry matter determination, 40 ml of the culture was passed through a 0.45-μm-pore-size filter, dried at 70°C for 24 h, and weighed.

Chemical analyses. During growth, 5- to 10-ml samples were removed under sterile conditions and centrifuged at 10,000 × *g*. The resulting pellet was used for starch and sugar determinations, and the supernatant was used for the assay of dissolved polysaccharides.

(i) Total sugars. The harvested cells were hydrolyzed in sulfuric acid (1 N) in a boiling water bath for 1 h. Cell debris was removed by centrifugation, and total sugars were determined in the supernatant by the phenol-sulfuric acid method (6).

(ii) Starch. For starch determination, a modification of the method of Haissing and Dickson (10) was used. The algae pellet was extracted twice with methanol-chloroform-water, 12:5:3, to remove free sugars. The remaining material was then hydrolyzed with amyloglucosidase (no. A7255; Sigma Chemical Co., St. Louis, Mo.) in 0.1 M acetate buffer, pH 4.7. The concentration of the glucose obtained was measured with Sumner reagent (20).

(iii) Cell wall polysaccharide. The polysaccharide fraction bound to the cell wall (bound fraction) was determined by subtracting the starch content of the cell pellet from its total sugar content. For the determination of cell wall polysaccharide dissolved in the medium (dissolved fraction), the sugar content of the cell-free medium was analyzed by the phenol-sulfuric acid method. The total cell wall polysaccharide is thus the sum of the bound and dissolved fractions.

Polysaccharide characteristics. (i) Collection. Dissolved polysaccharides were collected from the medium of cultures grown for 4 to 6 weeks. The supernatant was dialyzed to remove salts, frozen, and lyophilized. The resulting powder was used for the following analyses.

(ii) Uronic acids content. The uronic acids content was estimated by the *meta*-hydroxydiphenyl method (1).

(iii) Sulfate content. Sulfate content was determined after hydrolysis of the polysaccharide sample with 2 M HCl for 2 h at 100°C, according to the method of Terho and Hantiala (22).

(iv) Anion exchange chromatography. To estimate the density of surface charges in the polysaccharide, anion-

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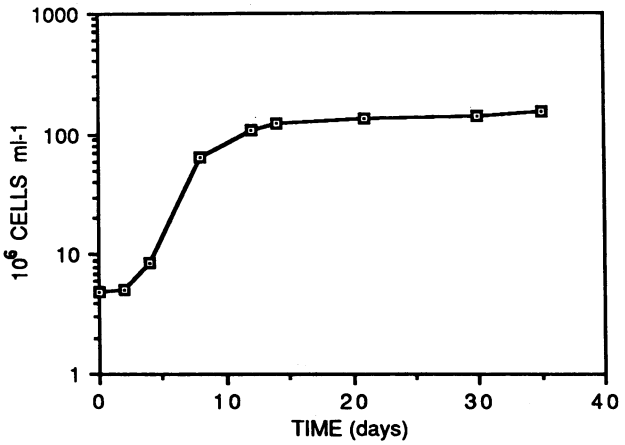

FIG. 1. Growth curve of *C. stigmatophora* LB 993.

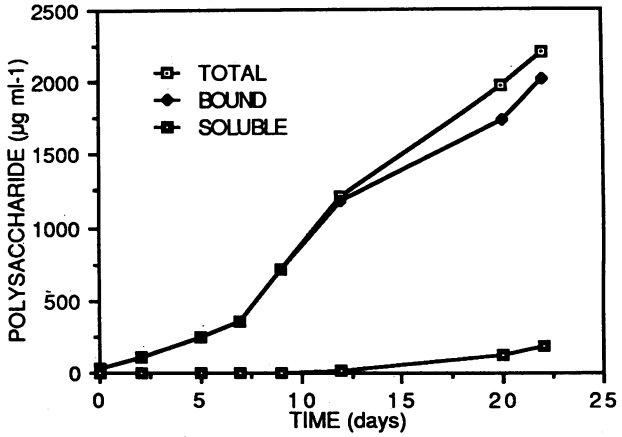

FIG. 2. Polysaccharide production in *C. stigmatophora* during growth.

exchange chromatography was performed on a column of DE 52 (Whatman, Inc., Clifton, N.J.). The elution was obtained by successively passing distilled water (neutral fraction) and then a discontinuous gradient of increasing concentrations of NaCl solutions (0.5 M; 1 M; 2 M; 5 M) and 6 M urea. Carbohydrates in the eluates were monitored with phenol-sulfuric acid reagent (6).

(v) Metal-complexing capacity. Dissolved polysaccharides were collected from the medium by centrifugation of cultures grown for 4 to 6 weeks on artificial seawater without EDTA. The cell-free medium was analyzed by anodic stripping voltammetry (ASV) either directly or after being dialyzed, lyophilized, and dissolved in double-distilled water. Essentially, ASV is a two-step electroanalytical technique. In the first step, the metals are deposited from solution into a small-volume mercury electrode (plating or preconcentration step) at a constant potential, and in the second step the metal is electrooxidized from the mercury. Differential pulse anodic stripping voltammetry was used for simultaneous determination of Zn^{2+}, Cd^{2+}, Pb^{2+}, and Cu^{2+} concentrations in samples by using a hanging mercury drop electrode (working electrode type E 410; Metrohm, Arefau, Switzerland). The analysis consists of the following steps: (i) plating the electrode at −1,400 mV for 120 s in stirred solutions flushed with CO_2; (ii) rest period, unstirred and unflushed for 30 s at the deposition potential; (iii) scanning from −1,400 to +300 mV and recording the current; (iv) stripping the working electrode at +300 mV in stirred and flushed solution; (v) starting again from (i). A fresh mercury drop was used for each plating-stripping cycle. Calibration was done by standard addition of increasing concentrations of the cations to each sample. All analyses were done at pH 4.5. More details of the analytical system were previously described (D. Kaplan, D. Raphaeli, and S. Ben-Yaakov, Talanta, in press; D. Kaplan, A. Abeliovich, and S. Ben-Yaakov, Water Res., in press). This technique permits simultaneous determination of the concentration of several dissolved free cations in the presence of their soluble salts and organic chelates (8, 19). The metal-complexing capacity of the tested solution was determined by standard addition of cation in question and monitoring the change in the ASV calibration curve. The total amount of the cation required to reach the break point represents the chelating capacity of the original solution (W. R. Matson, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1968) (19). All the chemicals used were of analytical grade. Appropriate blanks were run for all of them, and only the corrected values are presented.

RESULTS

The production of polysaccharides by *C. stigmatophora* LB 993 was monitored throughout the growth of batch cultures. Under the conditions used, the maximum cell number was 150 × 10^6 cells ml^{-1}, and the culture entered the stationary phase of growth, after 8 days (Fig. 1). During the logarithmic phase of growth, the doubling time of the culture was 1.4 days.

Changes in the production of the polysaccharide during growth of *C. stigmatophora* (both the bound and the dissolved fractions) are shown in Fig. 2. Attached polysaccharide was found to increase from the inception of culture growth, whereas the dissolved fraction was detected only after day 12, that is, after cell division had ceased. Most of the polysaccharide produced remained attached to the cell wall and the dissolved fraction accounted for only about 10% of the total polysaccharide.

The ability of dissolved polysaccharides of *C. stigmatophora* to bind Zn^{2+}, Cd^{2+}, Pb^{2+}, and Cu^{2+} was studied for

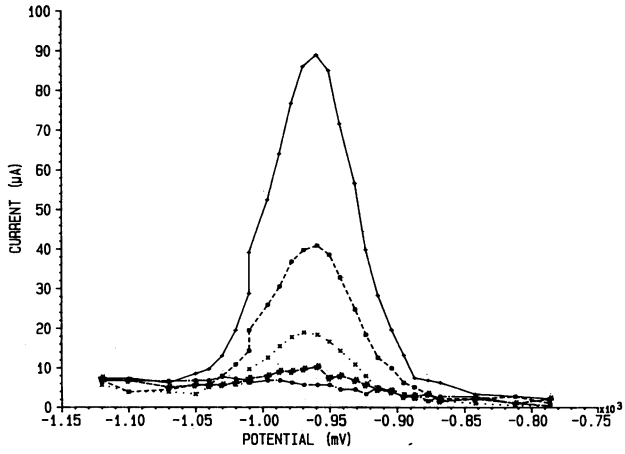

FIG. 3. ASV voltammogram of Zn^{2+} added to dissolved polysaccharide (45 µg of glucose equivalent ml^{-1}) of *C. stigmatophora*. Symbols: ●, 0; #, 200; ×, 250; ■, 300; +, 400 ppb of Zn^{2+}.

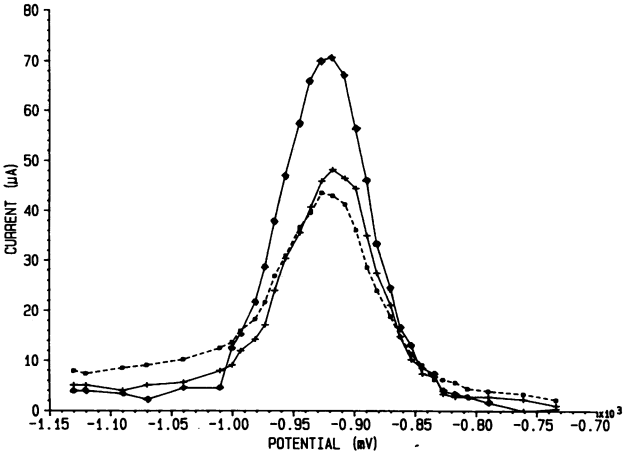

FIG. 4. ASV voltammogram of Zn^{2+} added to dissolved polysaccharide (48 μg of glucose equivalent ml^{-1}) of *C. salina*. Symbols: ■, 0; +, 10; ◆, 80 ppb of Zn^{2+}.

algae growing in EDTA-free medium. Figure 3 presents a typical voltammogram of Zn^{2+} added to dissolved polysaccharides produced by *C. stigmatophora* grown for about 4 weeks. Free Zn^{2+} was not detected in the medium in which *C. stigmatophora* grew (Fig. 3). Upon gradually increasing the amount of zinc added, a typical peak of Zn^{2+} at a potential of −960 mV appeared, with the signal increasing proportionally to the amount added.

The capability of two other marine species of *Chlorella*, *C. salina* and *C. luteoviridis*, to release polysaccharides to the growth medium was tested. Polysaccharides were detected in growth medium from cultures of the former species but not of the latter. However, the dissolved polysaccharides obtained from *C. salina* failed to bind heavy metals. A typical voltammogram of Zn^{2+} added to dissolved polysaccharide produced by *C. salina* is shown in Fig. 4. Free Zn^{2+} was detected in the original sample, and the signal for Zn^{2+} increased when the amount of metal added to the sample was gradually raised.

The relation between increasing the dose of Zn^{2+} added and the current peak height in cell-free medium of *C. stigmatophora* and *C. salina* is shown in Fig. 5A and B. The

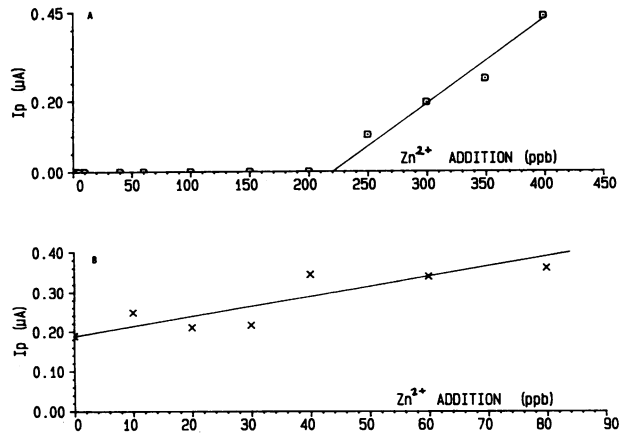

FIG. 5. Titration curve of current peak height versus Zn^{2+} concentration of (A) *C. stigmatophora* and (B) *C. salina*. (Polysaccharide concentration as described in the legend to Fig. 3 for A and Fig. 4 for B.)

TABLE 1. Complexing capacity of dissolved polysaccharide from *C. stigmatophora* for Zn^{2+}, Cd^{2+}, and Cu^{2+} as a function of polysaccharide concentration

Cation	Complexing capacity (ppb) of polysaccharides (in μg of glucose equivalent ml^{-1})			
	10	15	30	45
Zn^{2+}	None	None	165	215
Cd^{2+}	None	80	200	310
Pb^{2+}	None	None	15	30
Cu^{2+}	160	325	510	>1,000

shape of the curve in Fig. 5A indicates formation of a complex between the compound(s) in the sample and the Zn^{2+}. As long as the metal is bound by the polysaccharide, no increase in current peak height is observed; once the complexing agent(s) sites are saturated, addition of metal raises the current peak height. For the specific sample presented, obtained from *C. stigmatophora* (Fig. 5A), the complexing capacity for Zn^{2+} is about 213 ppb (213 ng/ml). For the sample from *C. salina*, the curve is linear (Fig. 5B), and the concentration of free Zn^{2+} is 78 ppb.

The dissolved polysaccharide of *C. stigmatophora* was found to be capable of binding Cd^{2+} and Cu^{2+} besides Zn^{2+}; the binding of Pb^{2+} was negligible (Table 1). The metal-complexing capacity of these polysaccharides was found to be different for each of the metals. The amount of metal bound also depended on the concentration of polysaccharide in the solution.

The binding capability might be due to negative surface charges of the polysaccharide that possibly result from the presence of uronic acids or sulfate (or both) on the polysaccharides. Thus, the content of uronic acids and sulfate in the dissolved polysaccharides of *C. stigmatophora* and *C. salina* was compared. The uronic acids content was about three times higher in *C. stigmatophora* than in *C. salina*, around 30% of the polysaccharide in the former species and 9% in the latter; the sulfate content was 10 and 7%, respectively.

To find the degree of heterogeneity of the dissolved polysaccharide obtained from each alga, it was fractionated by using anion-exchange chromatography. The elution pro-

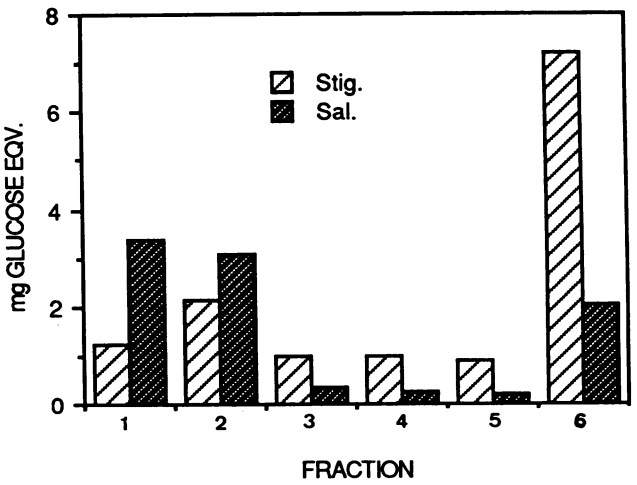

FIG. 6. Elution profile of the polysaccharide of *C. stigmatophora* and *C. salina* on a DE-52 anion exchange column. Fractions: 1, double-distilled water; 2, 0.5 M NaCl; 3, 1.0 M NaCl; 4, 2.0 M NaCl; 5, 5.0 M NaCl; 6, 6.0 M urea.

files of the DE-52 anion exchange chromatography (Fig. 6) show that the polysaccharides of *C. stigmatophora* and *C. salina* are heterogenous polymers. Neutral and weakly charged fractions (eluted in double-distilled water and 0.5 M NaCl) represented about 70% of the polymer of *C. salina* and only 25% of that of *C. stigmatophora*. By contrast, charged fractions (from 1 M NaCl to 6 M urea) constituted the major portion of the polymer mixture (75%) in *C. stigmatophora*, most of it being eluted in 6 M urea only.

DISCUSSION

The results presented in this study demonstrate that the capability of algal polysaccharides to bind heavy metals is rather specific. Not all the polysaccharides excreted by algae exhibit metal-complexing capacity. Moreover, as demonstrated here, various algal species within the same genus, although producing polysaccharides, do not necessarily bind heavy metals. Indeed, a correlation between highly anionic charged polymers and metal-complexing capacity was found. It seems that the free carboxylic groups (contributed by uronic acids) and their relatively homogenous distribution in the polysaccharide of *C. stigmatophora* play a major role in metal complexing. Sulfate ions apparently play a minor role, since no specific binding was exhibited by *C. salina* polysaccharide, which contains a concentration of sulfate similar to that of *C. stigmatophora* but a much lower uronic acids content. The correlation between negative-surface-charged polymers resulting from the existence of higher quantities of uronic acids and metal-complexing ability found here is supported by the observation that alginates extracted from seaweeds and bacteria exhibit Cu^{2+}-binding ability, as does pectate, a polymer of β-D-galacturonic acid found in plant tissue (17). Similarly, bacterial extracellular polysaccharides with uronic acids and hexosamines containing negative surface charge also bind metals (2, 5, 7).

The study of binding of heavy metals by algal polysaccharides for reducing metal toxicity in bodies of water is of potential ecological importance. These polymers may be used either as natural chelates or as part of biological water purification processes.

The dissolved polysaccharides whose binding characteristics were studied are only a small portion of the cell wall polysaccharides. It is assumed that the structure of both the dissolved and the bound fractions is similar, and that the cell surface can serve as a metal chelator. However, more information is required concerning cell wall structure and composition in relation to binding properties.

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