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Production of Exocellular Polysaccharide by *Azotobacter chroococcum*

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ABSTRACT

Environmental conditions affect the production of extracellular polysaccharide by *Azotobacter chroococcum* ATCC 4412. Production of exocellular polymer from a variety of carbon sources depended on the air flow rate. A high sucrose concentration in medium (8%) markedly favored exopolysaccharide production, which reached 14 g/L in about 72 h. In cell suspensions incubated in the presence of 8% sucrose in a nitrogen-free medium, biopolymer final concentration of 9 g/L corresponds to 68 g/g biomass. Maximum efficiency of sucrose conversion into exopolysaccharide peaked at 70% for initial disaccharide concentration of 6%. High performance liquid chromatography and gas liquid chromatography of acid hydrolysates of the exopolymer revealed the presence of mannuronosyl, guluronosyl, and acetyl residues, but not neutral sugars. The infrared spectrum corroborated the presence of carboxylate anions and *O*-acetyl groups in the exopolymer. Though the presence of more than one kind of polysaccharide cannot be ruled out, these data suggest that, under the experimental conditions used in this work, only a type of alginate-like exopolysaccharide is produced by *A. chroococcum* ATCC 4412.

Index Entries: Alginate; carbon sources; biotransformation; molasses; *Azotobacter chroococcum*.

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INTRODUCTION

Some microbial polysaccharides are commercially useful for producing gels and modifying the rheological properties of aqueous systems. Since these biopolymers can be readily available independent of sunlight and climates, they have the potential to replace exopolysaccharides from plants and marine algae traditionally used in the food, pharmaceutical, textiles, and oil industries (*1*). Microbial alginate is the product of two groups of bacteria, *Azotobacteriaceae* and *Pseudomonadaceae* (*2–5*). Bacterial alginate is an acetylated polymer of D-mannuronic and L-guluronic acids, and evidence has been presented for the location of *O*-acetyl groups on some of the D-mannuronosyl residues (*1*).

Most studies on *Azotobacter* alginate have been carried out with the species *A. vinelandii,* both wild and mutant strains, and it was claimed that bacterial alginate production could be cost-effective (*6*).

Though in the pioneering work by Lawson and Stacey (*7*) the existence of two capsular polysaccharides in *A. chroococcum* was reported, only recently have these two polysaccharides been characterized definitively (*8*). One of these exocellular polysaccharides was identified as an alginate. Interestingly, not only the overall yield and the relative amounts of the two exopolysaccharides, but also their chemical composition was found to vary from batch to batch, thus explaining the conflicting results from previous studies on the exopolysaccharide from *A. chroococcum* (*8,* and refs. therein).

Although we did not study its chemical composition, production of an alginate-like polysaccharide by *A. chroococcum* was greatly stimulated under nitrogen starvation (*9*). Secretion of alginic acid by *A. vinelandii,* however, was found to be maximal in a phosphate- and *N*-rich medium (*10*). We report now on the effects of some environmental factors and medium composition on the production of exopolysaccharide by *A. chroococcum.* A preliminary account of this work has appeared elsewhere (*11*).

MATERIALS AND METHODS

Microorganism

A. chroococcum (strain ATCC 4412 from the Valencia University Collection, Valencia, Spain) was used throughout this study.

Growth Media

Stock cultures of bacteria were maintained on slopes containing a combined nitrogen-free medium (*12*) supplied with 2% (w/v) sucrose as the sole carbon and energy source and solidified with 2% (w/v) Difco

agar. A preculture of *A. chroococcum* was made by inoculating 100 mL of the nitrogen-free growth medium with an appropriate amount of stock culture in a 250-mL conical flask and shaking it at 30°C under air in a gyratory shaker at 200 rpm. After 10–12 h, an appropriate volume of this cell suspension was transferred into 100 mL liquid medium in a 200 mm height ×40 mm diameter glass tube with pointed bottom and narrow (20 mm) mouth, and air at a rate of 147 L/L culture/h was then bubbled through it. Mid-exponential phase cells (absorbance at 560 nm of approx 0.5) were harvested by centrifugation at 1000*g* for 10 min, washed with medium free of bound nitrogen, and resuspended to a cell density of about 50 μg of cell protein/mL of the same medium ("dinitrogen-fixing cells"). Unless otherwise stated, cell suspensions that had been subcultured at least twice under the conditions to be assayed were used as inocula. Where indicated, an Ar:O_2 (79%:21%) gas mixture substituted for the air atmosphere. When nitrate-grown cells or ammonium-grown cells were used, the combined nitrogen-free medium was supplemented with 8 m*M* KNO_3 or NH_4Cl, respectively.

All the liquid cultures used in this work were 2.5 m*M* in potassium phosphate buffer, pH 7.5. The buffering capacity was maintained by supplementing the media with 50 m*M* MOPS (3-(*N*-morpholino)propanesulfonic acid)/KOH buffer, pH 7.5.

Polysaccharide Assay and Bacterial Dry Weight Determination

A 40 mL sample of cell suspension was centrifuged at 24000*g* for 20 min at 4°C. Sedimentation was improved by adding NaCl and EDTA to final concentrations of 100 m*M* and 10 m*M*, respectively. The supernatant was precipitated with 3 vol of propan-2-ol and washed with a mixture of propan-2-ol:water (3:1). The exopolysaccharide was determined after drying in an oven at 90°C to constant weight. The cell sediment was washed once with water, and cells were then collected by vacuum filtration on glass microfiber paper from Whatman and dried to constant weight at 90°C. For all analytical work, the precipitated polysaccharide was redissolved in distilled water and lyophilized.

Analytical Procedures

The infrared absorption analysis of *A. chroococcum* exopolysaccharide was carried out in a Perkin-Elmer 457 IR on samples included in KBr disks. Uronic acids in the exopolysaccharide were identified by both thin layer chromatography in silica gel (Whatman) K6 of neutralized acid hydrolysate and by gas/liquid chromatography (Hewlett Packard 5710 A) of the corresponding alditol acetates (*13,14*).

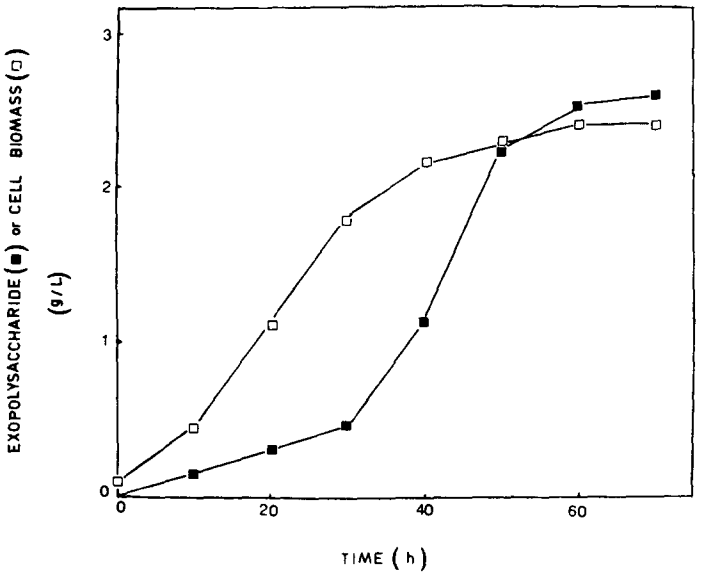

Fig. 1. Time-course of exopolysaccharide production by *A. chroococcum*. (■), Exopolysaccharide; (□), bacterial dry wt.

Sucrose, glucose, and fructose were determined following enzymatic methods (*15*). All the results are representative of at least three separate experiments with different batches of bacteria. Where indicated, variability in the data is expressed as percentages of variations between these replicates.

RESULTS

Polysaccharide Production

Exopolysaccharide production by *A. chroococcum* was followed in batch fermentation for 70 h at 30°C. Figure 1 shows that during growth of the organism in Burk's medium with sucrose (20 g/L) as the sole carbon and energy source, production started at a low rate early during growth. Exponential growth ended at approx 30 h, whereas exopolysaccharide production increased markedly at this time and continued maximally for 20 h during the stationary phase before decreasing.

The production of exopolysaccharide by *A. chroococcum* was influenced by the medium composition. Table 1 shows that exopolysaccharide production was stimulated by increasing the air flow rate from 32 to 147 L/L culture/h, and that an increase in aeration up to 225 L/L culture/h caused a decrease in yields of polysaccharide and bacterial dry wt.

Cultivation temperature (20, 25, 30, 35, and 40°C) was investigated. Table 2 indicates that the highest biopolymer concentration (2.4 g/L) was

Table 1
Effect of Air Flow Rate
on Exopolysaccharide Production by *A. chroococcum*

Air flow rate (L/L culture/h)	Bacterial dry wt[a] (g/L)	Exopolysaccharide[a] (g/L)
32	2.1	1.5
80	2.4	2.2
147	2.7	2.4
225	1.8	1.7

[a]The variations of the bacterial biomass formed and exopolysaccharide produced between three separate experiments did not exceed ±12%.

Table 2
Effect of Incubation Temperature
on Exopolysaccharide Production by *A. chroococcum*

Temp (°C)	Bacterial dry wt[a] (g/L)	Exopolysaccharide[a] (g/L)
20	1.6	1.2
25	2.1	1.9
30	2.3	2.4
35	1.8	1.8
40	0.9	0.7
45	No growth	—

[a]Determined at 72 h from inoculation. The variations of the bacterial biomass formed and exopolysaccharide produced between three separate experiments did not exceed ±15%.

obtained when cells were cultivated for 72 h at 30°C, which was also the optimum temperature for cell growth (2.3 g bacterial dry wt/L).

Exopolysaccharide production (2.2 g/L) by cells grown under N_2-fixing conditions was slightly higher than in nitrate-grown cells (1.5 g/L) or ammonium-grown cells (1.6 g/L). Changing the amount of phosphate in the medium down to 1 m*M* had an insignificant effect on polysaccharide production.

The effect of a variety of carbon substrates (2% w/v) on polysaccharide production by *A. chroococcum* was investigated. N_2-fixing cells were used to favor exopolysaccharide production. As shown in Table 3, the organism was able to synthesize exopolysaccharide from different mono- and disaccharides and sugar beet molasses carbon substrates. Sucrose and molasses were good growth substrates and gave the highest polysaccharide yields. Although glucose, fructose, and mannitol were relatively good growth substrates, they were less satisfactory as polysaccharide producers. Mannose and the nonsugar carbon substrate sodium acetate did not support growth.

Table 3
Effect of Carbon Source
on Exopolysaccharide Production by *A. chroococcum*

Carbon source[a]	Bacterial dry wt[c] (g/L)	Exopolysaccharide[c] (g/L)
Sucrose	2.8	2.3
Glucose	2.2	0.7
Fructose	1.9	1.0
Mannose	No growth	—
Mannitol	1.9	1.0
Acetate	No growth	—
Molasses[b]	3.5	2.4

[a]Carbon sources were at 2% (w/v).
[b]Sugar beet molasses was added in the amount corresponding to its total carbohydrate content (60%).
[c]Determined at 72 h from inoculation. The variations of the bacterial biomass formed and exopolysaccharide produced between four separate experiments did not exceed ± 15%.

Table 4
Effect of Sucrose Concentration on Exopolysaccharide and Biomass Production and on the Efficiency of Sucrose Conversion into Exopolysaccharide

Sucrose concentration (g/L)	Exopolysaccharide produced (g/L)	Cell biomass produced (g/L)	Sucrose used up[a] (g/L)	Yield (g biopolymer/ 100 g sucrose)
20	1.8	2.0	6.0	30
40	4.0	1.8	9.0	44.4
60	9.5	1.5	13.4	70.1
80	11.8	1.1	33.7	35

[a]Calculated as the difference between the sucrose added and the glucose, fructose, and sucrose determined in the medium at the end of the incubation time, which was 48 h.

The influence of sucrose concentration on exopolysaccharide and biomass production, and on the efficiency of sucrose conversion into exopolysaccharide, was studied in a series of batch cultures containing 2, 4, 6, and 8% (w/v) sucrose in medium. It could be ascertained that by increasing sucrose concentration in the medium up to 8%, production of exopolysaccharide by the bacterium increased whereas cell biomass drastically decreased. In the experiment described in Table 4, incubation of *A. chroococcum* cells for 48 h with 2% sucrose yielded 2.0 g bacterial dry wt/L and 1.9 g exopolysaccharide/L. A progressive increase in exopolysaccharide production was observed when sucrose concentration in medium was gradually raised from 2 to 8%. As shown in Table 4, the opposite was true for bacterial biomass, which diminished when sucrose augmented. With

Table 5
Effect of Nitrogen Starvation on Exopolysaccharide Production by *A. chroococcum*

Time (h)	Dinitrogen-fixing cells		Dinitrogen-starved cells	
	(g/L)	(g/g biomass)	(g/L)	(g/g biomass)
0	0.4	8.4	0.4	8.6
24	8.0	7.8	4.5	32.0
48	12.8	6.2	7.8	77.8
72	14.2	6.5	9.0	68.2

14-h-old N_2-fixing cells were collected, washed, and suspended in Burk's nitrogen-free medium (containing 8% sucrose) to 50 μg protein/mL and, at zero time, they were bubbled either with air ("Dinitrogen-fixing cells") or an Ar:O_2 gas mixture ("Dinitrogen-starved cells"). The variations of the exopolysaccharide formed between three replicates did not exceed ±15%.

8% sucrose, the figures were 1.1 g bacterial dry wt/L and 10.8 g polysaccharide/L during the same time-period.

Since we had found an exocellular invertase that accounted for most of the activity present in a cell-free homogenate preparation from *A. chroococcum* (*16*), to compute the yield of polysaccharide on basis of sucrose added, the analysis of monosaccharides, besides sucrose, was essential. Table 4 indicates that the highest efficiency of sucrose conversion into polymer was observed when the initial sucrose concentration was 6%. At the end of the incubation time, 9.5 g/L exopolysaccharide had been produced and, though not shown, a small amount of sucrose (1 g/L) remained in the cell-free supernatant. Twenty-five grams per liter glucose and 20 g/L fructose were detected in this supernatant, however. Therefore, the efficiency of sucrose conversion into exopolysaccharide was approx 70%.

It is well known that, in general, an increase in the cellular C/N ratio results in an enhanced microbial biopolymer synthesis. When *A. chroococcum* cell suspensions incubated in the presence of 8% sucrose were subjected to nitrogen starvation, exopolysaccharide production increased gradually with time, reaching approx 60% (9 g/L) of that attained by cells bubbled with air (Table 5). When the exopolysaccharide produced was expressed on a bacterial dry wt, the yield was rather high, about 70 g/g biomass, because no significant growth occurred. In fact, the cell density that had been established at zero time (A_{560} of about 0.4) held practically constant during the experiment.

Characteristics of the Exopolysaccharide

The infrared spectrum of the exopolysaccharide exhibited absorption bands at 1610/cm and 1408/cm, which indicates the presence of carboxylate ion (*17*), and a shoulder at 1633/cm, which suggests the presence of *O*-acetyl ester (*7*) (Fig. 2).

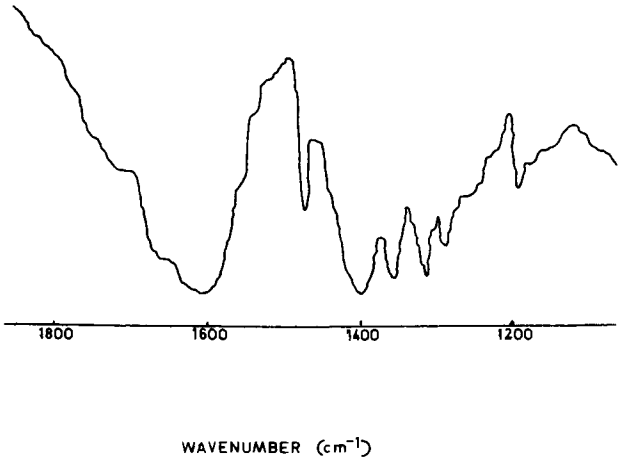

Fig. 2. IR spectrum of *A. chroococcum* exopolysaccharide.

Thin layer chromatography of the polymer acid hydrolysate did not show the presence of either galacturonic acid nor glucuronic acid, but a spot corresponding to mannuronic acid was seen. Another spot presumably pertaining to a second uronic acid was present. HPLC of an appropriate acid hydrolysate revealed the presence of mannuronic acid, a marked peak of acetyl residue, and an unidentified compound with retention time very close to that of mannuronic acid and that was neither glucuronic acid nor galacturonic acid. No neutral sugars were detected. To characterize the unidentified uronic acid residue seen by HPLC, the alditol acetates of the polysaccharide components were prepared and analyzed by gas liquid chromatography. The alditols corresponding to D-mannopyranosyluronic acid and L-gulopyranosyluronic acid residues were eluted with retention times of 12.62 and 15.93 min, respectively, the latter residue being analyzed as glucitol.

On the basis of these results, the unidentified uronic acid peak in the HPLC chromatogram is most likely guluronic acid. Considering the areal ratio of the peaks corresponding to acetate (4.1%), guluronic acid (11.8%), and mannuronic acid (25.3%), a minimal 1:3:6 ratio can be estimated for these three components of the *A. chroococcum* ATCC 4412 exopolysaccharide. The presence of mannuronosyl and guluronosyl residues and *O*-acetyl groups in this polysaccharide, as well as its infrared spectrum, strongly suggests that it could be alginic acid.

DISCUSSION

With the exception of the early work by Lawson and Stacey on the chemical characterization of the *A. chroococcum* acid capsular polysaccharide (*7*), and the recent report by Cote and Krull that the exocellular poly-

saccharide from *A. chroococcum* ATCC 7491 is comprised of two fractions, one of which is an acetylated alginate (*8*), information about exopolysaccharides from this organism is very scarce. Much less attention has been paid yet to the influence of the physicochemical environment on the production and quality of *A. chroococcum* exopolysaccharides.

We have found that the amount of extracellular polysaccharide formed by *A. chroococcum* ATCC 4412 depended on the culture conditions. From the viewpoint of commercialization, it was interesting to find that, among a variety of carbon source tested, sugar beet molasses supported both good growth and exopolysaccharide production. However, sucrose was routinely used for physiological studies in this work and no attempt to correlate the composition of the polymer synthesized and the carbon and energy source employed was undertaken.

Polymer formation by *A. chroococcum* batch cultures did not parallel growth. In fact, exopolysaccharide biosynthesis markedly increased at the end of the exponential growth and continued maximally during part of the stationary phase. Alginic acid production by *A. vinelandii* has been shown either to be concomitant with growth (*17*) or to increase at its cessation (*6*). In the same context, production of commercially useful xanthan (*1*) and other microbial exopolysaccharides (*18*) is closely linked to the total biomass. Exopolysaccharide production by *A. chroococcum* was highest when air flow rate was 147 L/L culture/h. A lower or higher flow rate was unfavorable. Though we did not measure the dissolved oxygen tension, these results agree well with those reported previously by Horan et al. (*19*), Deavin et al. (*20*), and Chen et al. (*6*), who have indicated that the amount of alginate produced was dependent on the oxygen tension. As a matter of fact, Horan et al. (*19*), using continuous cultures of *A. vinelandii*, found that the alginate biosynthesis rate increased when the dissolved oxygen tension varied from 0 to 5% and decreased above the later value. Interestingly, they could demonstrate that two of the key enzymes involved in alginate biosynthesis, i.e., mannosephosphate isomerase and GDP-mannose pyrophosphorylase, followed the same pattern as alginate in response to the dissolved oxygen tension. Furthermore, the activity levels of glucokinase and fructokinase increased also with the oxygen tension, and it could explain the observed increment in substrate utilization capacity displayed by the cells.

Polysaccharide production increased by using a favorable carbon:nitrogen ratio and, in this context, dinitrogen-fixing cells produced more polysaccharide than nitrate or ammonium-grown cells. Using dinitrogen-fixing cells and by increasing the concentration of carbon source in the medium up to 8%, yields as high as 14 g exopolysaccharide/L in about 72 h were obtained. This figure doubles the one achieved by other authors with *A. vinelandii*, which has been considered cost-effective (*6*), and it is a little lower than the productivity reported by Hacking et al. (*21*) using nitrogen-limited, *Pseudomonas mendocina* continuous culture containing 90 g

glucose/L. As it has been described in this work, the rise of polymer production promoted by increasing sucrose concentration was paralleled by a fall in cellular dry wt. This seems to suggest that, in *A. chroococcum* ATCC 4412, exopolysaccharide biosynthesis and cell growth compete for the carbon source. In fact, Okabe et al. (*17*) pointed out the same sort of competition in an *A. vinelandii* mutant. With the later strain, however, alginate biosynthesis was favored in the presence of low sucrose concentrations, which resulted in a much poorer cell growth. It is worth noting that competition for the carbon source was hypothesized to take place at the level of essential intermediates, probably polyisoprenoid cofactors, involved both in the exopolysaccharide synthesis and the cell wall synthesis (*22*). This proposal was difficult to maintain, at least for continuous culture, when it was described as the independence of rate of exopolysaccharide synthesis and specific growth rate in *A. vinelandii* (*23*).

Cells incubated in a nitrogen-free medium with 8% sucrose produced exopolysaccharide at an appreciable rate. Though, in absolute terms, the biopolymer produced was 60% of that attained under N_2-fixing conditions, when expressed on a bacterial dry weight basis the yields was as much as 68 g exopolysaccharide/g cell biomass.

The efficiency of sucrose conversion into polysaccharide deserves a special comment. With the exception of xanthan fermentation, in which 75% of sugar consumed by *Xanthomonas campestris* was converted into xanthan gum (*1*), the yield of bacterial exopolysaccharide based on sugar added is around 30% (*6,20,24*). As mentioned above, the finding of an exocellular invertase in *A. chroococcum* cells suspension (*16*) made it mandatory to analyze the medium for monosaccharides content, besides sucrose, in order to determine the efficiency of sucrose conversion into polysaccharide. The specific yield of exopolysaccharide produced, 70%, calculated after substracting the measured monosaccharide concentrations, was rather high in comparison with the values in the literature, which did not take into consideration this calculation. The rise in both exopolysaccharide production and yield with the increase of sucrose is most likely related to the invertase activity found in the extracellular medium.

As stated earlier, alginic acid has been recently identified as one of the two components of the exocellular polysaccharide of *A. chroococcum* isolated by Cote and Krull (*8*). The other component characterized by them, which consists of rhamnose, mannose, and galactose, along with pyruvic acetal and acetic acid groups, is apparently not formed under the growth conditions used in the present work. Our data, however, do not prove that we have only one polysaccharide for no fractionation and analysis of the fractions has been attempted. Unlike Cohen and Johnstone (*25*), who identified galacturomic acid as the main component of the exopolysaccharides of three strains of *A. vinelandii*, no galacturonic acid was detected in the preparation studied in the present work.

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