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Production and purification of an extracellular polyglucan produced by *Cellulomonas flavigena* strain KU

C.S. Buller and K.C. Voepel

Department of Microbiology, University of Kansas, Lawrence, KS, U.S.A.

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SUMMARY

A polysaccharide is synthesized by *Cellulomonas flavigena* strain KU when it is cultured in a synthetic medium which uses ammonium salts as a nitrogen source and contains an excess of a carbon and energy source. Production of the polysaccharide begins in late log phase and reaches a maximimum during stationary phase. In batch cultures it may be produced in yields of up to 9 g dry polysaccharide/liter of culture. It is not secreted into the growth medium but rather remains associated with the cells, resulting, apparently, in their aggregation. When such aggregated cells are extracted with dilute sodium hydroxide solutions the polysaccharide is solubilized. Neutralization of supernatant fluid of such extracts results in sedimentation of the polysaccharide which may then be purified by extensive washing with water. The polysaccharide is insoluble in water, alcohols or acetone, but dissolves in concentrated formic acid, dimethyl-sulfoxide, and dilute sodium- or potassium hydroxide. Thin-layer and gas-liquid chromatographic analysis of hydrolysates indicated that it is a polyglucan. When resuspended in water at concentrations of 2–3% it forms a stable hydrogel.

INTRODUCTION

Many bacteria have the ability to synthesize polysaccharides and to excrete them into the growth medium either as soluble or insoluble polymers. Exopolysaccharides may be a component of the bacterial glycocalyx [4], a bacterial structure which frequently is associated with adherent bacteria and bacteria newly isolated from their natural habitat [5]. *Cellulomonas flavigena* strain KU cells, when grown in minimal medium containing an excess of glucose, strongly adhere to each other. We have previously shown that the development of such cell aggregation temporally coincides with the deposition of capsules, and that the encapsulated cells contain a polysaccharide polymer which can be utilized as an energy reserve [17]. In this communi-

Correspondence: C.S. Buller, Department of Microbiology, University of Kansas, Lawrence, KS 66045, U.S.A.

cation the growth conditions which lead to the production of that polysaccharide polymer, the procedure for its extraction and purification, and its characterization as a polyglucan are reported.

MATERIALS AND METHODS

Bacteria

Cellulomonas flavigena strain KU has been described [2]. Other species of *Cellulomonas* were obtained from the American Type Culture Collection (Rockville, MA).

Culture media and growth of bacteria

The minimal salts culture medium, designated as CM9, was of the following composition (g/l): KH_2PO_4, 3.18; K_2HPO_4, 5.20; $MgSO_4$, 0.12; yeast extract (Difco), 0.50. NH_4Cl was used as a nitrogen source and was added at various concentrations, as indicated in the text. Five milliliters of heavy metal solution [16] was added to the minimal medium to insure the availability of metals which commonly are used as cofactors. The pH of the medium was adjusted to 6.8. Solutions of the carbohydrates that were used as energy sources were sterilized separately.

Cultures were prepared either as shake cultures, using baffled Erlenmeyer flasks containing media in an amount not in excess of 0.25 of the rated capacity of the flasks, or in a Braun Biostat M table top fermentor. Cultures were incubated at 30°C, and flask cultures were aerated by incubation with shaking at 250 rpm. The fermentor cultures were 1 liter in volume. They were aerated at a rate of 1.6 l/min and were stirred at a rate of 400 rpm. The pH was continuously monitored and automatically maintained at 6.7. The amount of acid produced by such cultures was monitored by recording the amounts of 1 N NaOH pumped in to maintain the pH at 6.7.

Measurement of cell mass

The dry weight of bacteria in a culture was determined after washing the cells in water and drying them to a constant weight in an oven in which temperature was maintained at 60°C.

Reducing sugar analysis

The reducing sugar content of washed, dry cells and of purified polysaccharide was determined by the phenol-sulfuric acid assay, using glucose as standard [9].

Extraction and purification of polymer

The procedure used for recovery and purification of the polysaccharide polymer is outlined in Fig. 1. Recovery and washing of the aggregated bacteria was facilitated by their rapid sedimentation. Thus, after allowing cultures to stand briefly the cell-free upper phase could be removed by aspiration and the remaining cells collected by centrifugation. After several water washes, the bacterial cells were resuspended in 1.0 N NaOH, at a concentration of 1 g (wet weight) of cells/4 ml of 1.0 N NaOH. Wet weight determinations were based upon the weights of precipitates obtained after centrifugation of suspensions of washed cells for 15 min at 15 000 × *g*. Two extractions were sufficient for the removal of at least 95% of the polysaccharide. Neutralization of the supernatant fluids from the first and second extractions, using either 50% (w/v) acetic acid or 6

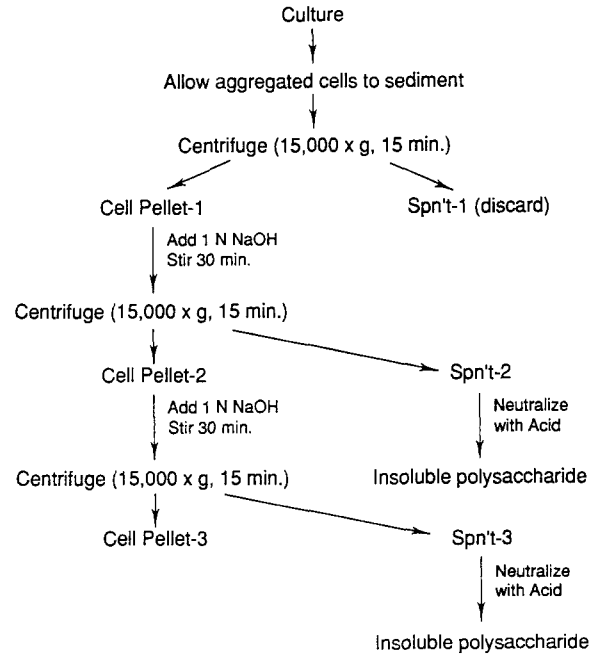

Fig. 1. Procedure for extraction and purification of a polysaccharide from *Cellulomonas flavigena*.

N HCl caused the polysaccharide to separate out of solution and in the process the entire volume of the supernatant fluid was congealed. Such hydrogels were then resuspended in 10 to 20 volumes of water and, after stirring, the water insoluble polysaccharide was allowed to settle out. The clear upper phase was then removed by aspiration. This process was repeated until the preparation was free of inorganic phosphate and protein.

Chromatography

The polysaccharide, resuspended at a concentration of 2 mg/ml of 2 N H_2SO_4 in sealed tubes was hydrolyzed at 100°C for ca. 5 h. The hydrolysates were neutralized with barium carbonate, filtered, and then, after concentration in vacuo, analyzed by thin layer chromatography (TLC) and gas liquid chromatography (GLC).

TLC was performed as described by Stahl and Kaltenbach [13] using silica gel HR-boric acid plates and methylethyl ketone-acetic acid-methanol (60:20:20) as the solvent system. After development the plates were dried and sprayed with aniline phthalate to detect sugars.

For GLC analysis the sugars were converted to trimethylsilyl (TMS) derivatives, using Tri Sil Z (Pierce, Rockford, IL). Analysis was performed with a Varian Aerograph 1740 equipped with a flame ionization detector, using a 6 ft stainless steel column 1/8 in. in diameter packed with 3% SP-2250 (50:50 methyl:phenyl silicone) on 80/100 Supelcoport. Column temperature was 185°C and N_2, at a flow rate of 22 ml/min, was used as the carrier gas.

Analytical methods

Protein content was estimated by the method of Lowry [11], and phosphate by the procedure described by Ames [1].

RESULTS

Effect of nitrogen concentration on biomass and polysaccharide production

The synthesis of bacterial exopolysaccharides often is enhanced if the organisms are grown in a medium containing limiting amounts of nitrogen and an excess of the carbon and energy source [6,15,18,19]. The relationship of the nitrogen concentration of the growth medium to the growth of *C. flavigena* strain KU and the amount of polysaccharide synthesized is shown in Fig. 2. In these experiments *C. flavigena* strain KU was grown in shake cultures. After incubation for 72 h samples were removed for the estimation of bacterial growth and of the amount of extractable polysaccharide. Because the cells in such cultures are densely aggregated it was not possible to use dilution-plating for the determination of cell numbers. Accordingly, growth was estimated on the basis of the wet weight of biomass, determined before extraction of the cells with NaOH. The estimates of polysaccharide production were based upon the amount of hydrogel which could be obtained from alkaline extracts of the bacteria. To standardize the determination of the wet weights the samples were always centrifuged for 15 min at 15 000 × *g*. Fig. 2 shows that an increase in the concentration of NH_4Cl, from 0.005 M to 0.015 M, was accompanied by an increase in biomass. Polysaccharide synthesis also increased, but the maximum yield was observed in the medium with 0.01 M NH_4Cl. The decrease in biomass and hydrogel at higher concentrations of NH_4Cl prob-

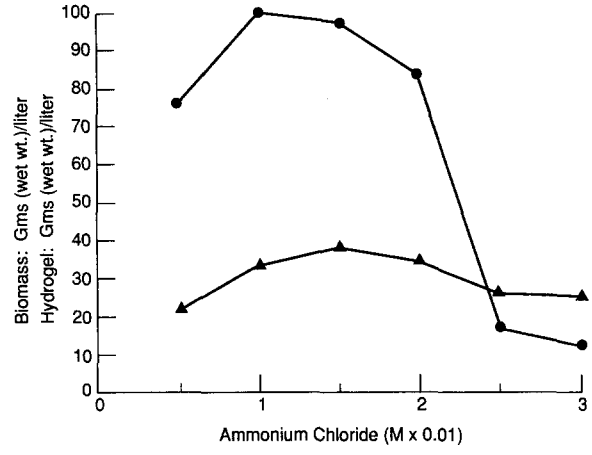

Fig. 2. Effect of nitrogen concentration on biomass and hydrogel production. Bacteria were grown in flask cultures in CM9 medium containing 0.05% (w/v) yeast extract plus the indicated amounts of ammonium chloride and 4% (w/v) glucose. Cultures were incubated with shaking at 250 rpm and at 30° for 72 h. Symbols: ▲, biomass; ●, hydrogel.

Table 1

Effect of nitrogen concentration on polysaccharide production

NH_4Cl (M)[a]	Final pH	Polysaccharide/ total biomass[b]
0.005	6.80	3.45
0.010	6.64	2.98
0.015	6.34	2.54
0.020	5.62	2.45
0.025	4.90	0.63
0.030	4.76	0.45

[a] Growth medium contained 0.05% yeast extract and 4% glucose. Cultures were incubated for 72 h at 30°C with shaking at 250 rpm.

[b] Ratio based upon wet weights of polysaccharide and biomass, determined after centrifugation for 15 min at 15 000 × *g*.

ably was a consequence of the accumulation of acid in the medium. *Cellulomonas* species are facultatively anaerobic and may produce considerable amounts of acid from glucose [3,13]. Table 1 shows that as the concentration of NH_4Cl in these growth media was increased there was a progressive decrease in both pH and in the polysaccharide hydrogel/wet weight cells ratio.

The validity of using the amount of hydrogel formed from alkaline extracts as an estimate of polysaccharide synthesis, as described above, is dependent upon a demonstration that the composition of the gel remains constant. This was examined by comparing the reducing sugar contents of hydrogels collected from bacteria grown under conditions which lead to variations in the ratios of wet weights of hydrogel/biomass. Bacteria were grown in CM9 medium containing 0.01 M NH_4Cl and 2% glucose. Thirty-milliliter aliquots of culture were removed at 24, 48, 72, and 96 h and the amounts of hydrogel formed from alkaline extracts of the cells in them was compared. In such experiments the ratio of hydrogel/biomass increased in a linear fashion from a value of 0.84 for samples removed at 24 h to one of 4.3 for those removed at 96 h. The hydrogels were then washed with H_2O, dissolved in 1 N NaOH, and their reducing sugar content determined. The reducing sugar content of the hydrogel varied only slightly, from 29.4 to 30.6 mg/gm (wet wt) of hydrogel. Similar results were obtained when the reducing sugar content of hydrogels extracted from cells grown for 72 h in CM9 medium containing 2% glucose and either 0.005, 0.01, or 0.015 M NH_4Cl. In cells from these cultures the ratio of hydrogel/biomass decreased from 3.6 for cells from the cultures containing the least NH_4Cl to 2.6 for those cells grown in a medium containing 0.015 M NH_4Cl.

The decrease in the polysaccharide/biomass ratio observed in cells grown in media with increasing NH_4Cl concentration (Table 1) presumably was a consequence of the low final pH of the culture medium. Accordingly, growth of the bacterium was examined in a 1-liter table top fermentor which provided automatic pH control. Fig. 3 shows the results from an experiment in which the amount of biomass in samples removed at the indicated times and the reducing sugar content of alkaline extracts of such samples was determined. The examination of the samples by phase contrast microscopy indicated that the cells had begun to aggregate at 22 h and by 24 h the extent of aggregation was such that the cells rapidly settled out of suspension. The final yield of biomass was 71.1 mg/ml (wet weight), in contrast to the yield of 34 mg/ml obtained in shake

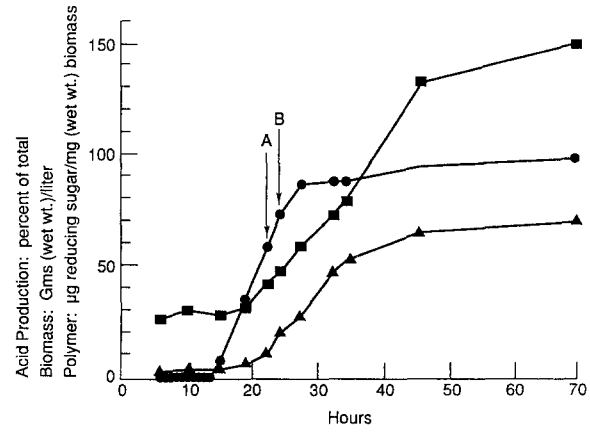

Fig. 3. Time course of growth and polysaccharide production. Bacteria were grown in a Biostat M table top fermentor with the pH maintained at 6.7. CM9 culture medium contained 0.02 M NH_4Cl and 2% (w/v) glucose. Symbols: ●, acid; ▲, biomass; ■, polymer; A, aggregation first observed by phase contrast microscopy; B, macroscopic aggregation.

cultures which also were 0.02 M with respect to NH_4Cl but which lacked pH control (Fig. 2). The dry weight yield of biomass, determined only on the final sample from the pH controlled culture, was 14.9 mg/ml.

Characterization of an alkali soluble polymer extracted from aggregated cells

The chemical composition, solubility characteristics of the polysaccharide and some properties of the hydrogel are listed in Table 2. For these determinations the bacteria were grown in shake cultures in CM9 medium containing 0.0075 M NH_4Cl and 2% (w/v) glucose. In such cultures growth eventually was limited by the low inorganic nitrogen content of the medium and consequently the pH did not fall below 6. After 72 h of incubation the aggregated cells were collected and the polysaccharide was extracted and purified. The composition of the hydrogel which was obtained in such a manner was 97% (w/w) water and 3% (w/w) polysaccharide. When heated slowly such gels became clear at about 60°C and upon continued heating again formed opaque gels. When the water of such hydrogels was removed by lyophilization, a white, fluffy residue remained. When the hydrogel was dried in a hot air oven the residue was brittle and glassy in appearance.

The chemical composition of acid hydrolysates of the polysaccharide was determined by TLC and GLC analysis. Thin layer chromatography of such hydrolysates revealed 1 spot which had an R_f identical to that of authentic glucose. A GLC analysis of TMS derivatives of the hydrolysate likewise indicated that the polymer is comprised of glucose only.

The results of quantitative analyses of the reducing sugar content of the polysaccharide also indicated that it is a polyglucan. In such assays the polysaccharide, after being dried to constant weight, was dissolved in 1.0 N NaOH. Appropriate dilutions, made with 0.05 N NaOH, were then quantitatively assayed for reducing sugar. The entire weight of the polysaccharide could be accounted for as reducing sugar if glucose was used as the reference sugar.

Production of the polyglucan by other Cellulomonas species

The results of comparisons of the production of polysaccharide by *Cellulomonas flavigena* strain KU to that by other species of *Cellulomonas* is shown in Table 3. With the exception of strain ATTC # 21399, a *Cellulomonas* of undesignated species, none were able to produce yields of polymer equivalent to that produced by the *C. flavigena* KU. The KU strain, although classified as *Cellulomonas flavigena*, differed from ATTC #482 in that thiamine

Table 2

Characteristics of the polyglucan

A. Chemical composition
   1. Glucose homopolymer
B. Solubility characteristics:
   1. Soluble in 0.1 N (or greater) sodium and/or potassium hydroxide.
   2. Soluble in 85% (or more concentrated) formic acid.
   3. Soluble in dimethyl sulfoxide (DMSO).
   4. Poorly soluble in ammonium hydroxide.
   5. Insoluble in water, methanol, ethanol, isopropanol, and acetone.
C. Properties of the hydrogel
   1. After centrifugation at 15 000 × *g* for 15 min 97% of the weight is accounted for by the entrained water.

Table 3

Production of polysaccharide hydrogel by *Cellulomonas* cultures

Bacteria	ATCC No.	mg bacteria/ml of culture (wet weight)	mg of hydrogel/mg of bacteria
C. flavigena KU	53703	41.6	3.67
C. sp.	21399	36.0	3.71
C. uda	491	21.0	2.45
C. flavigena	482	18.7	1.30
C. cartae	21681	9.7	0.0
C. gelida	488	8.1	0.0
C. fimi	15724	7.2	0.0
C. biazotea	486	7.1	0.0
C. fimi	484	6.9	0.0

Bacteria were grown in minimal medium containing 0.0075 M NH_4Cl and 1% (w/v) glucose. Cultures were incubated at 30°C, with aeration, for 72 h.

and biotin were not both required for growth, and also in its cellular morphology when grown under conditions that lead to aggregation of cells [2]. As indicated in Table 3 a further difference is that the KU strain produces much more polysaccharide polymer than does ATCC #482, which is considered to be the type species of *Cellulomonas flavigena* [13].

DISCUSSION

Production of an extracellular polysaccharide by *Cellulomonas flavigena* has not been reported previously. It may have been unnoticed because the bacteria do not produce it when they are grown in standard minimal salts media, such as M9, containing the usual 0.018–0.02 M NH_4Cl unless the glucose concentration is at least 1% (w/v). At higher concentrations of glucose the culture becomes so acidic that growth and polymer production are decreased.

Exopolysaccharide synthesis occurs in a variety of bacteria and in many instances the amount synthesized is at its maximum when the growth medium has limiting amounts of nitrogen and an excess of the carbon and energy source [6,15,18,19]. A similar relationship occurs with *C. flavigena* strain KU when cultured in shake flasks (Fig. 2). In such cultures, however, the amount of polymer produced may be related to the pH of the medium, which falls, presumably as a consequence of an increase in growth, as the concentration of nitrogen in the medium is increased. High yields of biomass and polysaccharide, however, can be obtained in media containing 0.02 M NH_4Cl if the pH is maintained at about 6.7 (Fig. 3).

Fig. 3 shows a temporal correlation between the onset of polysaccharide synthesis, the first observations of aggregation, and the rapid increase in wet weight biomass. Because of technical problems attendant to cell aggregation we did not attempt to determine the number of bacteria per ml, and therefore cannot identify the growth phases of the culture. We assume, however, that the production of acid occurs at its maximal rate during the log phase of growth of the culture and that its rate of production diminishes as the culture enters the stationary phase. If so, most of the polysaccharide accumulation occurs during the stationary phase of growth.

In stationary phase cultures of ATCC 482, the type species of *C. flavigena* [13], the cells become swollen and distended, apparently because of the development of a very large inclusion body [2]. Similar morphological changes were not observed in *C. flavigena* strain KU. The relationship, if any, of that morphological difference to the production of an extracellular polysaccharide is unclear at this time. The type species produces a polysaccharide when grown under conditions of nitrogen limitation and glucose excess, but the yield, when expressed as mg of hydrogel/mg of bacterial biomass, is only about 36% of that produced by *C. flavigena* strain KU. If expressed as mg of hydrogel extractable from the cells in 1 ml of culture the amount from the type strain is only 16% of that from the KU strain. This indicates differences in growth rates and in amounts of polysaccharide synthesized by the type species and the KU strain. Of the cultures of *Cellulomonas* held by the American Type Culture Collection, only ATCC # 21399 produced the polysaccharide in amounts similar to those of *C. flavigena* strain KU.

The preliminary analyses reported here indicate that the polymer is a polyglucan. The type of glucosidic linkage has not yet been determined. That the polymer is not hydrolyzed by amylases or cellulases is taken as tentative evidence that it is not an α-1,4- of a β-1,4-glucoside. In its solubility characteristics and its ability to form hydrogels it is similar to the curdlans [7,8]. Nakanishi et al. [12], however, tested a large number of organisms, including *Cellulomonas flavigena*, for the ability to produced curdlan, and found that only certain strains of *Alkaligenes faecalis* and of *Agrobacterium* were able to do so.

A more detailed investigation of the chemical and physical structure of the polyglucan is in progress and will be reported later.

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