Production of Extracellular Polysaccharide Matrix by Zoogloea ramigera

ALICE B. PARSONS and PATRICK R. DUGAN

Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

Received for publication 4 November 1970

Zoogloea ramigera 115 synthesized large amounts of matrix polymer from fructose, galactose, glucose, lactose, mannose, soluble starch, and sucrose when these carbohydrates were used as supplements to a chemically defined medium. All of them supported polymer synthesis to the extent that cultures thickened to a gel. Concentration of carbohydrate nutrients in the range 0.5 to 2.0% was not a critical factor in determining eventual total thickening to a gel, except in relation to the incubation time required. Glucose disappeared from the growth medium rapidly and correlated with increasing cell growth and poly-β-hydroxybutyrate (PHB) accumulation. PHB concentration decreased as extracellular polymer was synthesized, suggesting a link between PHB and extracellular polymer production.

**MATERIALS AND METHODS**

**Culture and media.** Z. ramigera 115 was employed in this study (6, 8). It was cultured and maintained in the following modification of the arginine medium of Crabtree et al. (2): arginine-HCl, 0.5 g; alanine, 1.0 g; MgSO₄·7H₂O, 0.2 g; K₂HPO₄, 2.0 g; KH₂PO₄, 1.0 g; carbohydrate, 5.0, 10.0, or 20.0 g; vitamin B₁₂, 1.5 × 10⁻⁶ g; distilled water to 1 liter. Fructose, galactose, glucose, lactose, mannose, soluble starch, and sucrose were examined individually as the carbohydrate source (0.5 to 2.0%) in the basal medium.

In each case, the polymer was isolated from 100 ml of culture incubated in 2.0% carbohydrate in basal medium after 7 to 14 days at 24 ± 2°C on a reciprocal shaker (120 strokes/min). To study changes in batch culture and growth medium parameters, 10 liters of the medium in a 14-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) was inoculated with a 1% volume of a 72-hr culture and maintained at 24 ± 2°C with continuous agitation (300 rev/min) and aeration (3 liters of air/minute) for 7 to 20 days.

**Isolation and purification of polymer.** In each case, polymer from the cells grown in carbohydrate source was extracted and isolated as outlined in Fig. 1.

Acid hydrolysis of dried extracellular polymer. One milligram of purified polymer and 1 ml of 2 N HCl were sealed in replicate 5-inch soft glass tubes and hydrolyzed at 100°C for either 8.5 or 18 hr. The hydrolyzed material was removed from the sealed tube and transferred to a 50-ml beaker which was held in a water bath at 90°C; volatile material was evaporated under a stream of air. A 1.5-ml sample of distilled water was added to the hydrolyzed residue and again evaporated. This procedure was repeated three times to completely evaporate the HCl; the residue was then redissolved in 0.3 ml of distilled water and stored under refrigeration for subsequent paper chromatography.
100-ml culture (7 to 14 days)

Add 2 volumes 1 N NH₄OH; blend 15 sec; centrifuge at 1,600 × g for 20 min

Supernatant (A)
Add 3 volumes of cold, slightly acidified 95% ethanol; chill at 0 C overnight; if polymer is floating, lift out with a watch glass; otherwise, centrifuge at 1,300 × g for 60 min

Pellet, Discard

Supernatant, Discard

Pellet (B)
Redissolve in 1/2 original volume of cold water overnight; centrifuge at 1,500 × g for 10 min.

Supernatant
Reprecipitate as in A above and repeat steps through B; (C) re-dissolve precipitate in as small a volume of water as possible; dialyze at 5 C for 72 hr in double-distilled demineralized water changed daily; reprecipitate with acidified ethanol or acetone (2 volumes); chill overnight; collect polymer and air dry; store in desiccator

Retention

FIG. 1. Flow diagram outlining procedure for extraction and isolation of polymer.

**Paper chromatography.** Descending paper chromatography with Whatman no. 4 paper strips (19 by 80 cm) and with a cylindrical glass tank equilibrated at 24 ± 2 C was used to identify polymer components in the hydrolyzed samples. Known sugars (100 μg/ml) and polymer hydrolysates (0.1 ml) were spotted on each sheet. The three solvent systems selected for use were: 1-butanol-acetic acid-water [4:1:5(v/v)], isopropanol-water [4:1(v/v)], and ethyl acetate-pyridine-water [12:4:5(v/v)]. Location reagents included naphthoresorcinol dip (0.2% in acetone, 1 volume to 1 volume of 9% phosphoric acid in water), silver nitrate spray (0.1 M AgNO₃ in 5 M NH₄OH), benzidine dip (0.1 g in 40 ml of glacial acetic acid, 30 g of trichloroacetic acid, and 40 ml of water, 1 volume to 9 volumes of acetone), and ninhydrin spray (1.0 g of ninhydrin in 500 ml of 1-butanol and 0.5 ml of collidine).

**Fermentor studies and viscosity determination.** At each sampling time approximately 150 ml of whole culture was removed from the fermentor. Two 10-ml samples of whole culture material were frozen at −70 C for subsequent determination of dry weight, total deoxyribonucleic acid (DNA; 3), and poly-β-hydroxybutyric acid (PHB; 9). Two 10-ml samples of supernatant (6,600 × g for 10 min, Sorvall SS-1) were frozen at −70 C for subsequent determination of Nelson's reducing sugar (1) and anthrone sugar (10). An 8-ml sample of whole culture and an 8-ml sample of the supernatant were used for viscosity determination to estimate polymer production. Precalibrated (No. 50 B801, No. 200 Y823, No. 500 392) Cannon-Fenske Routine Viscometer tubes (Cannon Instrument Co.) were held plumb in a water bath at 23 ± 3 C. Duplicate time determinations were recorded for each sample. The average time in seconds for each sample was multiplied by the temperature-corrected viscosity constant for each tube to obtain viscosity in centistokes.

**RESULTS AND DISCUSSION**

When Z. ramigera 115 is grown in liquid shake-flask, it becomes extremely viscous and then semisolid in the presence of 0.5 to 2.0% glucose. The flask can then be inverted without
disturbing the culture. This is referred to as total thickening or gelled culture. Rate of total culture thickening was observed to be dependent upon glucose concentration in the culture medium. Cells cultured in basal medium which contained fructose, galactose, glucose, lactose, mannose, soluble starch, or sucrose as the supplemental carbon source produced abundant and nearly equivalent amounts of polymer. Since the polymer synthesis increases as a function of the concentration of carbon source in the medium, it would be reasonable to assume that the organism would produce small amounts of polymer in low organic environments. It could then be surmised that the organism would be more prevalent in the aquatic habitat than previously thought but might not be recognized as Zoogloea because of lack of capsular material synthesis in the absence of high organic carbon nutrient concentrations.

Paper chromatograms of acid-hydrolyzed polymers isolated individually from cultures grown in various carbohydrates revealed only one carbohydrate spot when developed in isopropanol-water or in butanol-acetic acid-water as reported by Friedman et al. (7) and revealed two distinctly separate spots for glucose (Rg 1.00) and galactose (Rg 0.87) when developed in an ethyl acetate-pyridine-water system. These correspond with Rg values of known glucose and galactose in the same system. It was concluded that the polymer was a polysaccharide composed of glucose and galactose with glucose as the predominant sugar. Cells grown in a basal medium with fructose, galactose, glucose, lactose, mannose, soluble starch, or sucrose produced polymer consisting of only glucose and galactose.

Since the carbohydrate source did not affect the composition of the extracellular polymer,
glucose was selected as the carbohydrate source for all further investigations. The time-sequence of polymer production was investigated in a 14-liter fermentor. The cells were so firmly enmeshed in the matrix polymer that it was not possible to obtain cell counts. Therefore cell growth was followed by a total DNA determination.

Figure 2 gives the results of one study obtained from a 10-liter culture in 12 days. Utilization of glucose available in the medium is related to viscosity of both supernatant and whole culture sample and to accumulation of PHB. The level of glucose dropped in 58 hr from 62 to 4.0 μmoles and was therefore not available in the culture solution between 60 and 288 hr when the viscosity was observed to increase. PHB concentration rapidly increased between 20 and 40 hr and reached a maximum between 60 and 70 hr suggesting that PHB formation stopped when the glucose was spent. After 75 hr, there was a steady decrease in the quantity of PHB. At approximately 60 hr, viscosity of both supernatant and whole culture showed a marked rate of increase, suggesting a relationship between PHB expenditure and polymer synthesis. At 180 hr, an accelerated rate of increase in viscosity was observed. In the course of the study, the viscosity of the supernatant increased from 1.0 to 9.5 centistokes. In the same time-period, the viscosity of the whole culture sample increased from 1.0 to 680.0 centistokes. At this point, it became extremely difficult to centrifuge enough supernatant from the samples to allow viscosity measurement of the supernatant.

The rapid disappearance of glucose from the medium as determined by both anthrone and Nelson’s tests could be due either to rapid metabolism of glucose or to physical adsorption of the sugar by this organism. The bacterium has been shown to adsorb and accumulate metal ions and organic matter in its matrix, and the glucose could be adsorbed to the matrix polymer (5). Crabtree (Ph.D. Dissertation, Univ. of Wisconsin, 1966), studying a different isolate (I-16-M) of this organism, has associated the rapid initial uptake of glucose with the accumulation of PHB. In the present study, PHB accumulated within the cell; as the viscosity of the supernatant and of the whole culture increased, the quantity of the PHB decreased. Perhaps PHB is an intermediate storage compound that is utilized as a metabolite for extracellular polymer production or for synthesis of active precursors.

Figure 3 represents the results of a similar study over a period of 20 days. The DNA assay data approximate the familiar bacterial growth curve. As the total amount of DNA reached a

![Fig. 3. Viscosity of whole culture and supernatant, sugar in the medium, and total DNA in Z. ramigera 115 as functions of time. Growth was in 10-liter batch at 24 ± 2°C.](image)
maximum at 96 hr, over half of the available sugar had disappeared from the medium. After 250 hr, the reducing sugar present in the medium leveled off at 0.08 μmoles/ml or less. The total carbohydrate, as reflected by the anthrone test data, leveled off at 11 μmoles in the same time.

In this study, the viscosity of the culture supernatant increased from 1.0 to 10.3 centistokes. The viscosity of the whole culture sample, which contained cells and polymer, increased to 130 centistokes. In other batch cultures, the whole culture viscosity has been measured as high as 1,055 centistokes before total thickening made viscosity measurement physically impossible.

The above observations have been made on 17 different fermentor runs, and the following interpretations seem reasonable. After the cells had ceased to multiply (as suggested by the leveling off of the DNA), viscosity of both supernatant and whole culture continued to increase. This continued increase in viscosity could be due to increasing molecular weight, increasing branching, modification of the polymer (such as a change in water-binding properties), or further synthesis of more short-chain polymers. Figure 1 shows PHB still decreasing after 180 hr. The steadily increasing viscosity in the supernatants suggests the formation of a polymer precursor or low-molecular-weight polymer which remains in solution at 6,600 × g. The molecular weight of the polymer probably increases until it reverts from a soluble to a suspended molecule. Continued polymerization results in a matrix which flocculates from suspension and ultimately forms a gel. Viscosity in this situation can best be measured in whole culture samples to reflect increase due to both suspended and dissolved polymer.

The efficiency of glucose and arginine conversion to polysaccharide has been estimated from the data derived from several different fermentor studies. Cellular DNA was assumed to be 3% of the dry weight of the cells. Comparison to DNA growth curves indicated a production of 4.6 g of cells per liter at the point of total culture thickening. This value was compared to the total cell-floc dry weight value of 8.1 g per liter which was harvested from the culture vessel by centrifugation at the same time. The calculations indicate that the cells comprise about 55% of the total cell-floc dry weight. Total ash values in these experiments accounted for less than 6% of the dry weight, and it was assumed therefore that the extracellular polymer accounted for about 40% of the weight. If it is assumed that 50% of the cell dry weight and 40% of the extracellular polymer are carbon, we can account for 3.83 g of carbon per liter of a theoretical 4.22 g of carbon per liter added as glucose and arginine. The conversion of carbon substrate to cells is therefore estimated to be 54% and the conversion to polymer is approximately 34%. It has been previously reported that the floc-polymer adsorbs 99 times its own weight of water (4).

Z. ramigera 115 has the capacity to produce prolific quantities of extracellular polymer which can adsorb metal ions, organics, or both, from solution and can effect the removal of these materials from water by flocculation and settling processes. Increased knowledge of polymer production could lead to a workable system for industrial pollution abatement.

ACKNOWLEDGMENTS

This work was supported by grant no. WP00713 from the Federal Water Pollution Control Administration, U.S. Department of Interior.

We appreciate the technical assistance of Faith Reilly.

LITERATURE CITED

1. Clark, J. M. 1964. Experimental biochemistry, W. H. Freeman and Co., San Francisco.
2. Crabtree, K. T., E. McCoy, W. C. Boyle, and G. A. Rolich. 1965. Isolation, identification, and metabolic role of the sudanophilic granules of Zoogloea ramigera. Appl. Microbiol. 13:218–226.
3. Diache, Z. 1955. Color reactions of nucleic acid components. p. 235–305. In E. Chargaff and J. N. Davidson (ed.), The nucleic acids, vol. 1. Academic Press Inc., New York.
4. Dugan, P. R. 1970. Removal of mine water ions by microbial polymers. Proc. 3rd Symp. Coal Mine Drainage Res., p. 279–283. Mellon Institute, Pittsburgh.
5. Friedman, B. A., and P. R. Dugan. 1968. Concentration and accumulation of metallic ions by the bacterium Zoogloea. Develop. Ind. Microbiol. 9:381–388.
6. Friedman, B. A., and P. R. Dugan. 1968. Identification of Zoogloea species and the relationship to zoogloea matrix and floc formation. J. Bacteriol. 95:1903–1909.
7. Friedman, B. A., P. R. Dugan, R. M. Pfister, and C. C. Remsen. 1968. Fine structure and composition of the zoogloea matrix surrounding Zoogloea ramigera. J. Bacteriol. 96:2144–2153.
8. Joyce, G. H., and P. R. Dugan. 1970. The role of floc-forming bacteria in BOD removal from waste water. Develop. Ind. Microbiol. 11:377–386.
9. Law, J. H., and R. A. Slepecky. 1961. Assay of poly-beta-hydroxybutyric acid. J. Bacteriol. 82:23–36.
10. Steinbeck, C. C., and M. S. Rheims. 1959. A micromodification of the anthrone test for serum samples of limited quantity. Amer. J. Med. Technol. 25:377–380.