APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1976, p. 623-626
Vol. 31, No. 4

Printed in U.S.A.

Exopolymer Production and Flocculation by *Zoogloea* MP6

RICHARD F. UNZ* AND SAMUEL R. FARRAH[1]

Departments of Civil Engineering and Microbiology, Pennsylvania State University, University Park, Pennsylvania 16802*

Received for publication 3 November 1975

Flocculation by *Zoogloea* MP6 was accompanied by the production of a mucopolysaccharide exopolymer. Polymer formation was initiated in mid-logarithmic growth phase, and the quantity produced appeared to be influenced by the level of carbon and nitrogen in the culture medium.

Several mechanisms on microbial flocculation have been advanced and these are discussed in the review of Harris and Mitchell (8). One theory involves an exopolymeric bridging of cells to form aggregates or flocs. Previous investigators have alluded to a relationship between bioflocculation and the synthesis of polymeric substances (3, 4, 7). Certain microorganisms, e.g., *Zoogloea* spp., synthesize an insoluble exopolymer which envelops the cells to produce a Zoogloea. Exopolymer obtained from *Zoogloea* strains and activated sludge flocs have been chemically characterized, and amino sugars were found to be major components (12; S. R. Farrah and R. F. Unz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, G260, p. 63). In the present study, amino sugar was used as an indirect measure of the exopolymer formed by *Zoogloea* MP6 in experiments conducted to demonstrate the relationship, if any, between polymer production and flocculation.

Zoogloea MP6 was batch cultured in 1-liter volumes on a reciprocal shaker (80 strokes/min) at 20 C. The culture medium contained (per liter of distilled water): $(NH_4)_2SO_4$, 0.264 g; K_2HPO_4, 0.087 g; $MgSO_4$, 0.120 g; $CaSO_4$, 0.136 g; and sodium lactate, 1.00 g. Lactate has been used widely as a carbon and energy source for *Zoogloea* strains (6, 13, 14, 15). The medium was autoclaved for 15 min at 121 C and the final adjusted pH was 7.2.

Zoogloeal flocs were harvested from 50-ml aliquots of culture fluid by centrifugation. Flocs were washed twice in distilled water and boiled for 10 min in 0.02 M K_2HPO_4 to dissolve the exopolymer. Insoluble debris was separated from the polymer by centrifugation at 27,000 × *g* for 10 min. The clear supernatant was dialyzed against distilled water for 24 h at 5 C and concentrated with polyethylene glycol, if necessary. The polymer was precipitated by adding 0.2 g of cetyltrimethyl ammonium bromide per 25 ml of solution, and the precipitate was dried overnight at 5 C. The residue was then dissolved in 0.5 M NaCl and centrifuged at 27,000 × *g* for 10 min, and the supernatant was dialyzed against several volumes of distilled water over 24 h at 5 C. Purified exopolymer (approximately 0.5 mg/ml) and concentrated HCl were mixed to produce a 6 N HCl solution which was dispensed to screw-capped tubes. The tubes were held in a boiling-water bath for 45 min. The hydrolyzed polymer was liberated of HCl under a stream of air at 28 C and analyzed for amino sugar content by the modified Elson-Morgan colorimetric method as described by Kabat and Mayer (9), employing D-glucosamine·HCl as the standard. Contaminant-free exopolymer was indicated by the absence of absorption peaks at 260 nm (nucleic acids) and 280 nm (aromatic amino acid-containing peptides).

The level of flocculation achieved by *Zoogloea* MP6 under test conditions was estimated using optical density (OD) data obtained on similar unsettled and 4-h refrigerated, settled samples. For comparative purposes, an index of flocculation (IF) was calculated employing the formula:

$$IF = \frac{OD_{500}\text{ unsettled culture} - OD_{500}\text{ settled culture}}{OD_{500}\text{ unsettled culture}}$$

The IF formula is a modification of the percent dispersion equation used by others (1, 11); in this case larger quotients indicate relatively better floc formation.

Viable cell counts were determined by spread plating 0.1 ml of serially diluted samples on CY medium containing (per liter of distilled water): Casitone (Difco Laboratories, Detroit, Mich.), 5.0 g; yeast autolysate (Chas. Pfizer & Co., Inc.,

[1] Present address: Department of Virology and Epidemiology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77025.

New York), 1.0 g; and Ionagar No. 2 (Colab Laboratories, Inc., Glenwood, Ill.), 8.5 g. Incubation was at 23 C for 72 h. This medium permitted better recovery of cells than did solid sodium lactate-mineral salts medium.

Dry weight determinations were made on cell mass dried at 105 C for 24 h and cooled to constant weight.

Certain observations were apparent from inspection of the growth cycle data presented in Fig. 1. Viable cell counts and OD of the unsettled culture paralleled each other up to the stationary phase whereupon the OD decreased rapidly, owing to the coalescence of small flocs and resultant clarification of the culture medium. However, exopolymer production was initially detectable in mid-logarithmic growth at which time bacterial flocs appeared. A subsequent parallel increase was noted both in the ratio of amino sugar to cell mass and the magnitude of flocculation, which suggested that polymer production was linked to floc formation. Interestingly, polymer appeared to increase per unit weight of biomass well into the stationary phase without appreciable increase in IF values at the latter stages of growth. The unit increase must have taken place concomitant with a decrease in available carbon during growth. Hence, a second experiment was conducted to evaluate the effect of the carbon-to-nitrogen ratio (C/N) on amino sugar (polymer) and floc production. The constituents of the culture medium remained as previously described, except various combinations of the carbon and nitrogen sources were employed to give different carbon-to-nitrogen ratios. Sterile culture media in 50-ml volumes contained in 125-ml Erlenmeyer flasks were inoculated with 1.0 ml of a standard cell suspension (OD_{500} = 0.5) and incubated on a gyrotary shaker (150 rpm) at 20 C for 48 h. Following incubation, swirled contents of duplicate flasks were divided into approximately equal, volumetrically measured portions and concentrated by centrifugation. One portion from each flask was used for dry weight determination, and the other portion was used for amino sugar analysis.

It was found that the initial concentration of available carbon and nitrogen rather than the absolute C/N ratio affected polymer production (Table 1). For example, carbon-to-nitrogen ratios were the same for two of the media used, one of which contained 0.32 g of C per liter (as carbon source) and 0.11 g of N per liter (as nitrogen source) and the other which contained 0.16 g of C per liter (as carbon source) and 0.056 g of N per liter (as nitrogen source). However, mean amino sugar-to-dry weight of cell mass

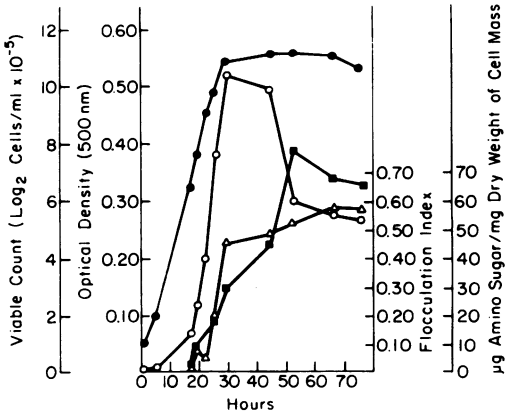

FIG. 1. *Growth, flocculation, and amino sugar production by Zoogloea MP6. Incubation at 20 C in sodium lactate-mineral salts medium. Symbols: ●, Viable cells; ○, optical density (500 nm); ■, amino sugar/dry weight of cells; △, flocculation index.*

ratios obtained for two experimental runs were 35 and 31 for the former culture medium and 57 and 50 for the latter medium. Assuming amino sugars comprise an average of 17% of the exopolymer (S. R. Farrah, Ph.D. thesis, Pennsylvania State Univ., University Park, 1974), the above amino sugar-to-cell mass ratios correspond to mean percent polymer content of approximately 19 and 32, respectively, for the two culture media. Non-nitrogenous exopolymer values of 20 and 40% of dry mass have been reported for *Zoogloea* strains (5, 10), and no information of a similar nature for amino sugar-containing exopolymer is available.

Our results suggest that while a deficiency in available carbon or nitrogen concentrations for growth will reduce the yield of exopolymer somewhat proportional to cell yields, the ratio of polymer to cells is maintained high under the condition of relatively low carbon, but not low nitrogen, concentrations. It is the ratio of polymer to cells that is considered important in the mediation of bioflocculation (2, 11), and availability of nitrogen appears critical for the synthesis of this mucopolysaccharide exopolymer.

A minimum level of polymer needed to initiate bioflocculation of *Zoogloea* MP6 cells was not determined, although most of the data indicated that effective flocculation (IF values > 0.4) occurred at amino sugar (micrograms)-to-biomass (milligrams) ratios of approximately 35:1 or greater. Very low available carbon may create a limiting growth condition for cells, causing a shunt of available carbon and nitrogen from protein synthesis to polymer production. In the activated sludge process of wastewater treatment, carbon is typically limiting at

TABLE 1. *Influence of carbon-to-nitrogen ratio on flocculation and amino sugar production by Zoogloea MP6*

Carbon as sodium lactate (g/liter)	Nitrogen as ammonium sulfate (g/liter)	Ratio of carbon to nitrogen	Run no.	Index of flocculation[a]	Dry wt of culture (mg/50 ml of culture fluid)[a,b]	Amino sugar (μg/50 ml of culture fluid)[a,b]	Ratio of amino sugar (μg) to dry weight of culture (mg)
0.32	0.55	0.6	1	0.50	10.9	440	40
			2	0.57	10.3	330	32
0.32	0.23	1.1	1	0.48	10.8	390	36
			2	0.64	9.5	340	36
0.32	0.11	2.9	1	0.62	13.2	460	35
			2	0.64	9.5	290	31
0.32	0.028	11.4	1	0.68	9.3	320	34
			2	0.62	8.2	235	29
0.32	0.011	29.1	1	0.31	5.1	80	16
			2	0.29	3.8	46	12
0.32	0.0028	114.3	1	0.03	1.7	20	12
			2	0.03	1.1	8	8
1.28	0.056	23.0	1	0.42	12.1	580	48
			2	0.64	8.5	350	41
0.64	0.056	11.4	1	0.50	5.6	280	50
			2	0.63	4.6	215	47
0.16	0.056	2.9	1	0.45	1.4	80	57
			2	0.48	0.9	45	50
0.08	0.056	1.4	1	0.32	0.6	30	50
			2	0.52	0.35	15	43

[a] Values represent average of duplicate samples.

[b] Values shown are the observed analytical results adjusted to reflect the contents in 50 ml of culture fluid.

the level of microorganisms (activated sludge) carried and may be the principal factor affecting exopolymer production by bacteria like *Zoogloea* spp.

This investigation was supported through grant 17050 DBI awarded by the United States Environmental Protection Agency.

LITERATURE CITED

1. **Angelbeck, D. I., and E. J. Kirsch.** 1969. Influence of pH and metal cations on aggregative growth of nonslime-forming strains of *Zoogloea ramigera*. Appl. Microbiol. 17:435–440.
2. **Busch, P. L., and W. Stumm.** 1968. Chemical interactions in the aggregation of bacteria bioflocculation in waste treatment. Environ. Sci. Technol. 2:49–53.
3. **Crabtree, K., E. McCoy, W. C. Boyle, and G. A. Rohlich.** 1965. Isolation, identification, and metabolic role of the sudanophilic granules of *Zoogloea ramigera*. Appl. Microbiol. 13:218–226.
4. **Deinema, M. H., and L. P. T. M. Zevenhuizen.** 1971. Formation of cellulose fibrils by gram-negative bacteria and their role in bacterial flocculation. Arch. Mikrobiol. 78:42–57.
5. **Department of the Environment.** 1973. Water pollution research 1972. Report of the director of water pollution research. Her Majesty's Stationery Office, London.
6. **Finstein, M. S.** 1967. Growth and flocculation in a *Zoogloea* culture. Appl. Microbiol. 15:962–963.
7. **Friedman, B. A., P. R. Dugan, R. M. Pfister, and C. C. Remsen.** 1969. Structure of exocellular polymers and their relationship to bacterial flocculation. J. Bacteriol. 98:1328–1334.
8. **Harris, R. H., and R. Mitchell.** 1973. The role of polymers in microbial aggregation. Annu. Rev. Microbiol. 27:27–50.
9. **Kabat, E. A., and M. M. Mayer.** 1961. Experimental immunochemistry. Charles C Thomas, Springfield, Ill.
10. **Parsons, A. B., and P. R. Dugan.** 1971. Production of extracellular polysaccharide matrix by *Zoogloea ramigera*. Appl. Microbiol. 21:657–661.
11. **Tenny, M. W., and W. Stumm.** 1965. Chemical floccula-

tion of microorganisms in biological waste treatment. J. Water Pollut. Control Fed. 37:1370–1388.
12. **Tezuka, Y.** 1973. A *Zoogloea* bacterium with gelatinous mucopolysaccharide matrix. J. Water Pollut. Control Fed. 45:531–536.
13. **Unz, R. F., and N. C. Dondero.** 1967. The predominant bacteria in natural zoogloeal colonies. II. Physiology and nutrition. Can. J. Microbiol. 13:1683–1694.
14. **Unz, R. F., and S. R. Farrah.** 1972. Use of aromatic compounds for growth and isolation of *Zoogloea*. Appl. Microbiol. 23:524–530.
15. **van Gils, H. W.** 1964. Bacteriology of activated sludge. IG-TNO Report no. 32. Res. Inst. Public Health Eng. The Hague, Holland.