APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1983, p. 1236–1238 Vol. 46, No. 5
0099-2240/83/111236-03$02.00/0

Influence of a Calcium-Specific Chelant on Biofilm Removal

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Received 31 May 1983/Accepted 1 September 1983

This paper describes the influence of ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA) on biofilm removal. The addition of EGTA resulted in the immediate detachment of biofilm which suggests that the chelant removed essential calcium from the biofilm, causing it to detach.

Biofouling is a general term referring to the undesirable attachment of microorganisms to surfaces of economic importance. Biofilms can increase fluid frictional resistance (12), heat transfer resistance (2), and the corrosion rate at the surface. Fouling of heat exchange equipment has been estimated to cost the United States billions of dollars annually (10).

Often, cleaning solutions containing metal ion chelants are used to remove inorganic scales which contain a considerable portion of divalent cations. These solutions probably remove bacterial films too, but there is no quantitative information available. The use of chelants to remove attached bacterial cells has been reviewed by Fletcher (6). In that study, examples were in quiescent conditions where the cells were not subjected to any fluid shear stress, and none used the calcium-specific chelant ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA). This paper describes experiments wherein buffered EGTA was used to detach a mixed bacterial film from the walls of a recycle tube reactor (Fig. 1).

The bacterial inocula for the experiments were prepared from a single batch of secondary aerobic sewage sludge from the treatment plant in Bozeman, Mont. The sludge was divided into 10-ml aliquots, frozen in 25% glycerol with liquid nitrogen, and stored at −20°C until needed.

The experimental system and medium composition are shown in Fig. 1. Medium and suspended cells from the mixing tank were recycled (flow rate $[F_R]$ = 7.5 liters min^{-1}) through the external loop of a pipe reactor at a rate considerably higher than the dilution rate (F_D), 0.065 liter min^{-1}, so that the system can be regarded as a continuous-flow stirred-tank reactor. Samples from the effluent were treated as follows. For glucose analysis, 5 ml of effluent sample was filtered (Nuclepore; average pore size, 0.45 μm) and the filtrate was frozen until used for analysis, using the Sigma 510 glucose analysis procedure. Suspended solids were measured by passing 25 to 50 ml of the effluent through a predried (103°C for 1 h) and preweighed Nuclepore filter (average pore size, 0.45 μm). After filtration, the filters were dried for 1 h at 103°C and reweighed. Total cell count was determined by using the epifluorescence procedure of Hobbie et al. (9). Effluent samples (5 ml) were fixed in 4% sterile Formalin, homogenized for 1 min in a Sorvall Omnimixer and refrigerated until the cell densities were estimated. Frozen 25-ml samples were thawed and analyzed for total carbohydrate, using the phenol reaction method of Hanson and Phillips (8). The fraction of suspended solids as suspended cells (milligrams [dry weight] per liter) was estimated by multiplying the epifluorescent cell count by the following factors: 1.963×10^{-13} cm^3 $cell^{-1}$; 1.07 g of cells cm^{-3} (1,4); 0.22 ratio of dry cell weight to wet cell weight (1, 11).

A biofilm was allowed to form over a period of several days, during which time suspended solids, suspended cells, total carbohydrate, and glucose were measured in the effluent. Glucose in the effluent was present as unused growth substrate and was never above 1 mg $liter^{-1}$ (i.e., more than 98% of the influent glucose was utilized by suspended and attached cells). Two hours before the addition of EGTA, sufficient Tris-hydrochloride buffer (pH 7.5) was added to the mixing tank to give a final concentration of 10 mM, and the dilution water was also buffered with 10 mM Tris (pH 7.5). Buffer addition did not perturb the system since no changes in the analytical characteristics of the effluent were detected. At time zero, sufficient EGTA (pH 7.5) was added to the mixing tank to give a concentration of 1 mM and was continuously fed to keep the concentration constant. EGTA addition caused no change in pH of the system.

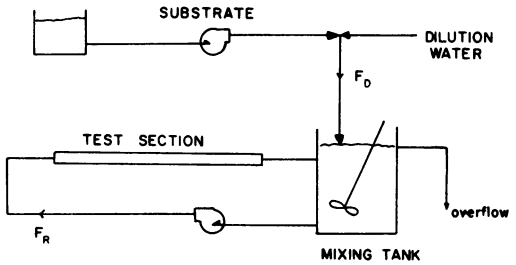

FIG. 1. A simplified flow diagram of a recycle tubular reactor (tube alloy A1-6X; influent glucose, 44.26 mg liter^{-1}; NH_4Cl, 0.159 mg liter^{-1}; $MgSO_4 \cdot 7H_2O$, 0.44 mg liter^{-1}; K_2HPO_4, 0.796 mg liter^{-1}; KH_2PO_4, 0.133 mg liter^{-1}.

A material balance approach was used to account for removal of biofilm from the tube surface. The mass balance (for suspended solids) across the reactor was as follows:

$$V\,dx/dt = -F_D x + R_D A \quad (1)$$

where $V\,dx/dt$ is the net rate of accumulation in the reactor, $-F_D x$ is the net rate of output by flow, and $R_D A$ is the net rate of detachment by chelant; V = volume of reactor (liters), A = surface area of reactor (meter^2), x = suspended biomass concentration (milligrams per liter), t = time (minutes), R_D = detachment rate (milligrams per meter^2 per minute), and F_D = dilution rate (liters per minute).

The increase in suspended solids by suspended cell growth and detachment of biofilm due to fluid shear stress and growth at the surface can be assumed to be negligible compared with the increase in suspended solids due to detachment by EGTA. Equation 1 can be rewritten as follows:

$$R_D = 1/A\,(V\,dx/dt + F_D x) \quad (2)$$

The detachment rate, R_D, in these experiments was calculated by using equation 2.

The addition of EGTA resulted in the removal of biofilm from the tube surface. The extent of detachment was monitored by measuring the increase in suspended solids, total suspended cells, soluble glucose, and total carbohydrate in the reactor effluent. The following observations were noteworthy.

The addition of EGTA resulted in removal of a major portion of the biofilm in the first 5 min. Figure 2 shows the increase in suspended solids in the effluent together with the detachment rate. Similar results were obtained in a second experiment, where the biofilm mass was approximately 10-fold greater than that in the first experiment. It seems likely that free calcium (or calcium associated with ligands of lower affinity than that of calcium for EGTA) is essential to the structural integrity of biofilm. The initial free

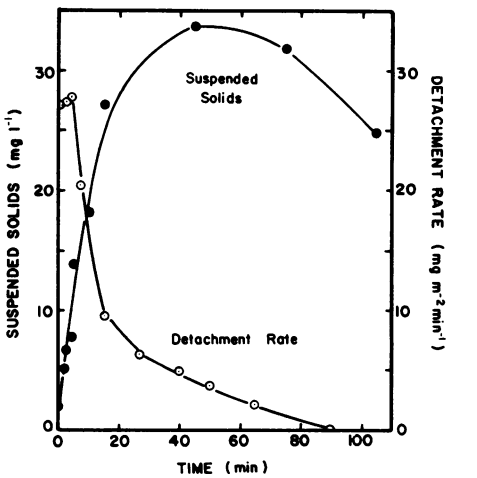

FIG. 2. Response of a biofouled tubular reactor to 1 mM EGTA (pH 7.5) addition at zero time (surface area of the reactor, 0.797 m^2; volume, 10.49 liters; residence time, 2 h).

calcium concentration was 0.6 mM and approximately 0.1 μM after EGTA addition.

The effluent-soluble glucose concentration did not rise on EGTA treatment, indicating that EGTA did not affect overall microbial substrate utilization and presumably microbial activity.

Calculated suspended cell mass was consistently much lower than the measured suspended solid mass (Fig. 3), which suggests that the biofilm is composed of material other than bac-

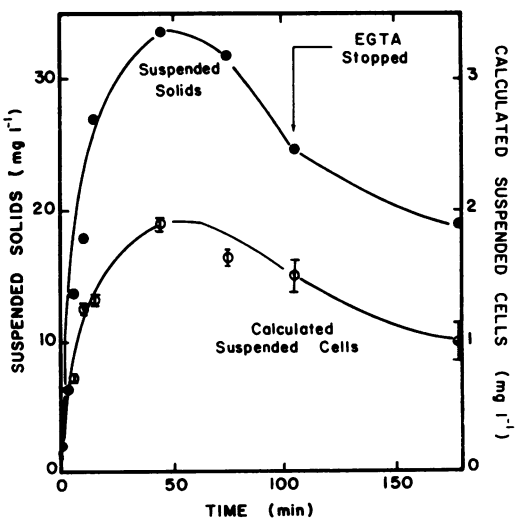

FIG. 3. Change in suspended solids and suspended cell mass in the effluent due to the addition of EGTA at zero time (error bars based on standard deviation from epifluorescent cell counts).

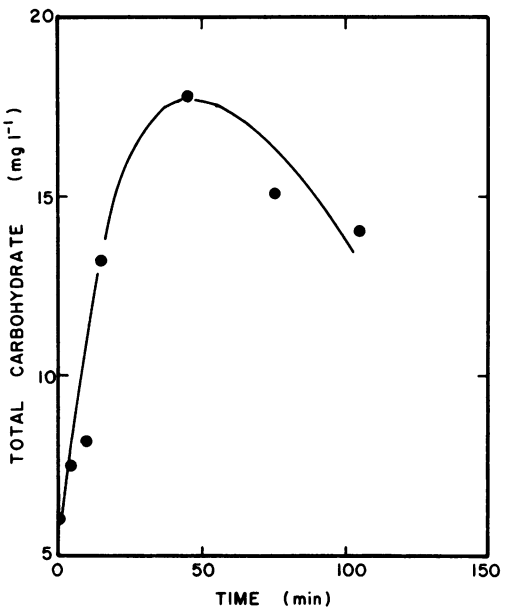

FIG. 4. Suspended total carbohydrate (as glucose equivalence) in effluent after addition of EGTA. Soluble effluent glucose concentration was less than 1 mg liter^{-1}.

terial cells. This is consistent with the finding of Trulear (Ph.D. thesis, Montana State University, Bozeman, 1983), who showed that biofilm is composed largely of extracellular polymer substances. Costerton et al. (3) and our results (Fig. 4) indicate that these substances are carbohydrates. Figure 4 indicates the change in effluent carbohydrate content following EGTA addition. Soluble glucose concentration during this period was less than 1 mg liter^{-1}. The data suggest that much of the detached material is composed of carbohydrates.

Fletcher and Floodgate (7) reported the disaggregation of extracellular polymer substances when bacterial flocs were transferred to medium deficient in calcium and magnesium. Transmission electron microscopic analysis showed the condensed nature of these substances after being fixed and stained (ruthenium red). We have performed experiments on biofilm scraped from the reactor and suspended in the growth media. Buffered EGTA was added to the suspended biofilm flocs under a phase-contrast microscope, and we observed disaggregation of the bioflocs (data not shown). Fletcher (6) considers that calcium may act as a cross-linking or charge-screening agent for ionic groups in the extracellular polymer substances. We suggest that calcium maintains the tertiary structure of these substances so that the interactions between the adjacent sugars on different chains are promoted. Spacing must be important in this respect. In previous experiments by Fletcher (5), lanthanum, an ion similar in radius to calcium but trivalent, did not substitute for calcium, although it binds at calcium sites less reversibly than does calcium in other systems (13). It is unlikely that calcium is involved in direct bridging to a negatively charged substratum since some bacterial cells remained on the internal surface of the tube even after EGTA treatment (data not shown). At the moment, we cannot rule out the participation of another attachment mechanism in bacterial adhesion which is unaffected by EGTA, especially since the work was performed in mixed culture.

We thank the United States Office of Naval Research (Oceanic Biology and Power Programs) for continued support of this research. W.G.C. also acknowledges partial support from the National Science Foundation, Chemical and Biochemical Systems Program, Division of Chemical and Process Engineering, and Calgon Corp.

J. A. Robinson, R. Bakke, and C. H. Nelson provided helpful advice.

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