Proceedings of the 12th Annual Biochemical Engineering Symposium

L. E. Erickson and L. T. Fan
Editors

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PROCEEDINGS OF THE TWELFTH ANNUAL BIOCHEMICAL ENGINEERING SYMPOSIUM

L. E. Erickson and L. T. Fan, Editors
Kansas State University
Manhattan, Kansas 66506

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This work presents the proceedings of the twelfth symposium which was held at Kansas State University on April 24, 1982. Since a number of the contributions will be published in detail elsewhere, only brief reports are included here. Some of the reports describe current progress with respect to ongoing projects. Requests for further information should be directed to Dr. Peter Reilly at Iowa State University, Dr. V. G. Murphy at Colorado State University, Dr. Rakesh Bajpai at University of Missouri, Dr. Ed Clausen at University of Arkansas, Dr. L. T. Fan and Dr. L. E. Erickson at Kansas State University.

The symposium was attended by Andrey Neryng, Peter J. Reilly, B. Douglas Brown, Judy A. Brown, LeAnn C. Shoenhard, Mike M. Meagher, and Zivko L. Nikolov of Iowa State University, Ravindranath Joshi, Jay Y. Lee, C. J. Huang, Mike Sierks, James C. Linden, Vincent G. Murphy, Timothy E. Maneely, and Michael Doremus from Colorado State University, Edward H. Hsu of Shell Development Co., Temkar M. Prakash and Rakesh K. Bajpai from University of Missouri at Columbia, Yong Hyun Lee of Purdue University, K. Toguchi of Mitsubishi Kakoki Co., Paul Sadler, Gary Magruder, Paul Doyle, and Ed Clausen of the University of Arkansas, and William H. Johnson, Mehmet Durdu Oner, Yan-fu Shi, L. T. Fan, Bumshik Hong, Bamidele O. Solomon, M. M. Gharpuray, J. R. Too, Satish K. Singh, Kyle Dybing, Shun-xi Rong, Kyu Chul Shin, C. H. Lee, Snehal A. Patel, Lie-chiang Chen, Tadayuki Ishimi, and Larry E. Erickson of Kansas State University.

L. T. Fan
L. E. Erickson
Editors

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PROGRAM

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KINETIC MODELING OF LIPID ACCUMULATION IN *Candida curvata R*

B. Douglas Brown and Kenneth H. Hsu

Department of Food Technology
Iowa State University
Ames, Iowa 50011

Introduction

Oleaginous yeasts are capable of accumulating intracellular lipid as 40-70% of their dry weight. Accumulation of this lipid is encouraged by aeration (Nilsson *et al.*, 1943) and by limiting nutrients such as nitrogen and phosphorus (Enebo *et al.*, 1946). Dual nutrient limitation of nitrogen and phosphorus improved lipid yields over limiting nitrogen alone or limiting nitrogen and magnesium (Gill *et al.*, 1977). Manipulation of the carbon to nitrogen ratio also effects the lipid yield (Hammond *et al.*, 1981). An excellent review is available (Ratledge, 1978).

In our lab, several strains of *Candida curvata* have been isolated which are capable of fermenting lactose in cheese whey permeate to lipid (Moon *et al.*, 1978). The production of lipid was observed to occur in three stages. In the first stage, growth in cell population was observed. In the second stage, nonlipid cellular growth ceased and lipid accumulation was apparently limited by available lactose. In the third stage, delayed whey protein utilization resulted in increases in nonlipid cellular mass. This occurred along with further conversion of lactose to lipid. We propose a model to describe the first two stages of this phenomena of intracellular lipid accumulation.

Model Development

Cellular dry mass, W, was subdivided into nonlipid dry mass, S, and lipid mass, F.

$$\frac{dW}{dt} = \frac{dS}{dt} + \frac{dF}{dt} \tag{1}$$

The specific growth rate of S, μ, was limited by nitrogen and described by the Monod equation.

$$\frac{dS}{dt} = \mu S = \frac{k_1 NS}{k_N + N} \tag{2}$$

Nitrogen was consumed solely for S production.

$$-\frac{dN}{dt} = \frac{1}{Y_1}\frac{dS}{dt} \tag{3}$$

Cell growth during this period was assumed balanced. The rates of lactose, L, consumption and F production were proportional to the production rate of S (Table I). Switching from balanced growth to lipid accumulation was proposed to be controlled by a power function of the relative specific growth rate of S, ϕ. More specifically, the function $(1 - \phi)^m$ was used. As N became depleted, ϕ would diminish, and terms containing the function (i.e., lipid accumulation terms) will become increasingly significant. Sensitivity of the change from growth to lipid accumulation to changes in ϕ was controlled by the parameter, m.

We proposed three models to describe the dependence of the rate of lipid accumulation on lactose (Table I). The first mechanism stated that only one enzymatic step leading from lactose to lipid was rate limiting (Model 1). The second mechanism described this single rate limiting step by nth order chemical kinetics (Model 2). The third mechanism proposed that two rate limiting enzymatic steps existed, resulting in the accumulation of an intermediate, I. The first enzymatic step was involved in the above proposed switching mechanism (Model 3).

This third mechanism was suggested by a biochemical mechanism for lipid accumulation in oleaginous yeast (Botham and Ratledge, 1979; Boulton and Ratledge, 1981). It was proposed that when N was limiting, the activity of the NAD+ dependent isocitrate dehydrogenase was curtailed through its absolute dependence on cAMP. As a result, citrate would be shunted to the cytosol where ATP:citrate lyase would apparently control the rate of lipid formation.

Methods

The three models were tested using data of batch growth of *Candida curvata R* on Swiss cheese whey permeate (Moon *et al.*, 1978). Graphical interpolation of the experimental points was used to generate sufficient data for the testing of the models Data from the first 10 hours were not used due to the phenomena of lag. After 50 hours, modeling was not attempted due to a significant consumption of previously unavailable N and synthesis of S. The delayed consumption of N was attributed to slow extracellular proteolysis of small proteins and peptides in whey permeate. For model fitting purposes N was set equal to zero after 30 hours.

The kinetic models, as a set of initial value, ordinary differential equations were integrated numerically. The parameters were determined by employing the Simplex search procedure (Fan *et al.*, 1969; Carpenter and Sweeney, 1965), through minimizing the residual sum of squares for N, L, F and W. The models were compared for best fit based on the ratios of the mean squares which has an F statistic distribution.

Results and Discussion

Table II summarized the parameter values obtained as a result of fitting the proposed models to the experimental data. The values of growth constants, and yield factors for models 1 and 3 were reasonable and reflected the fermentation kinetics. Model 3 gave a significantly better fit of the data than either model 1 or 2 (Table III). This indicated that the lipid accumulation mechanism could be better described using 2 rate limiting enzymatic steps. This observation is in agreement with the biochemical mechanism proposed by Boulton and Ratledge (1981). Model 3 is graphically compared with the experimental data in figures 1, 2, and 3. In figure 1, the model does not duplicate well the sigmoidal-shape curve of W. The sigmoidal shape seems to be due mostly to a fluctuation in the value of S between 30 to 45 hours.

The observed increase in the S fraction late in the fermentation is not common to all oleaginous yeast. Kessel(1968) observed a fairly constant non-lipid mass during the late stages of fermentation. Almazan *et al.* (1981) also observed in the second stage of a continuous fermentation, a constant percentage protein in the nonlipid cellular mass for the whole range of dilution rates. However, Choi *et al.* (1982) observed in a single stage continuous fermentation, a decrease in the percentage protein in the nonlipid cellular mass at very low dilution rates. Further experimentation will be necessary to verify which pehnomena is occurring in *Candida curvata*.

Comparisons of Models 1 and 2 with the experimental F and F/W data are given in figures 4 and 5. It is apparent that these models did not fit the experimental data as well as Model 3.

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TABLE I

Kinetic Models for Batch Growth of

Candida curvata R on Whey Permeate

Model No.	Kinetic Model
1	$\frac{dL}{dt} = \frac{-1}{Y_2}\frac{dS}{dt} - \frac{(1-\phi)^m}{Y_3}\frac{k_2 SL}{K_L + L}$; $\phi = \mu/k_1$
	$\frac{dF}{dt} = A\frac{dS}{dt} + \frac{(1-\phi)^m k_2 SL}{K_L + L}$
2	$\frac{dL}{dt} = \frac{-1}{Y_2 dt}\frac{dS}{dt} - \frac{(1-\phi)^m k_2 SL^n}{Y_3}$; $\phi = \mu/k_1$
	$\frac{dF}{dt} = A\frac{dS}{dt} + (1-\phi)^m k_2 SL^n$
3	$\frac{dL}{dt} = \frac{-1}{Y_2}\frac{dS}{dt} - \frac{(1-\phi)^m}{Y_3}\frac{k_2 SL}{K_L + L}$; $\phi = \mu/k_1$
	$\frac{dI}{dt} = (1-\phi)^m \frac{k_2 SL}{K_L + L} - \frac{k_3 SI}{(K_I + I)Y_4}$
	$\frac{dF}{dt} = A\frac{ds}{dt} + \frac{k_3 SI}{K_I + I}$

TABLE II

Comparison of Parameters for Kinetic Models

Parameter Value	Model Number		
	1	2	3
k_1	0.1942	0.1200	0.1944
K_N	0.1319	0.1704	0.1247
Y_1	38.25	42.98	38.56
Y_2	0.6401	0.3317	0.6892
A	0.1166	0.5325	0.1567
k_2	0.1420	7.060×10^{-7}	0.1517
K_L	66.24	---	59.11
n	---	4.702	---
Y_3	0.3019	0.3252	0.3518
k_3	---	---	2.476×10^{-2}
K_I	---	---	8.530×10^{-4}
$Y_4=0.3/Y_3$	---	---	0.8528
m	10.56	28.75	9.624

TABLE III

Results of Least Square Fit of Kinetic Models

Model No.	Number of Parameters	RSS	df[a]	MS	F
1	9	13.09	23	0.5691	2.086*
2	9	28.52	23	1.2400	4.545*
3	11	5.73	21	0.2729	------

[a]degrees of freedom = # data pts. - # of parameters

*$P < 0.05$

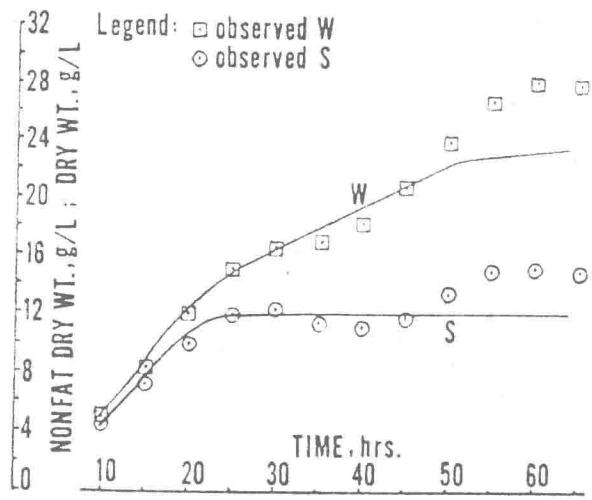

Fig. 1. Comparison of batch data with Model 3 for production of dry weight, W, and nonlipid dry weight, S.

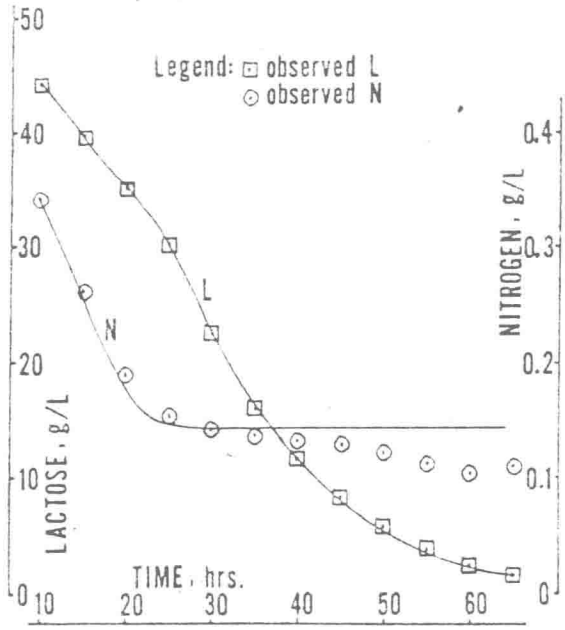

Fig. 2. Comparison of batch data with Model 3 for utilization of lactose, L, and nitrogen, N.

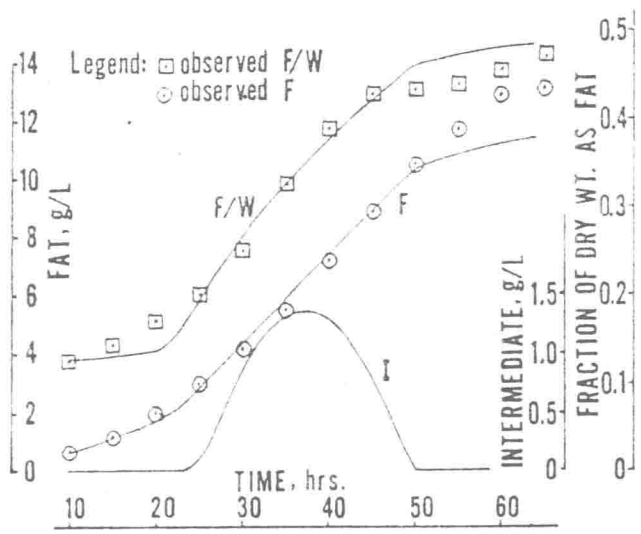

Fig. 3. Comparison of batch data with Model 3 for accumulation of intermediate, I, and production of lipid, F.

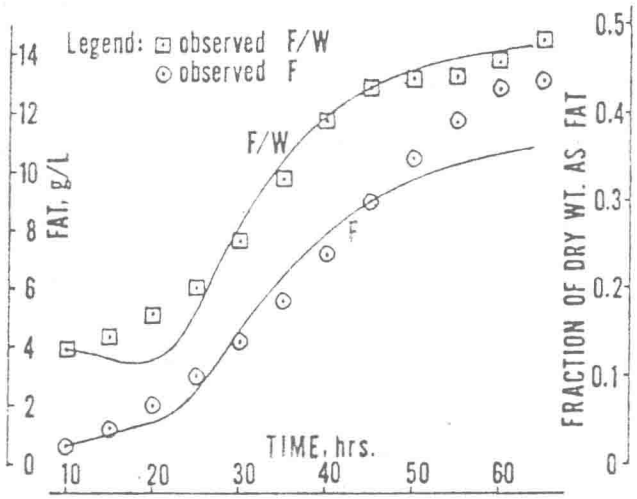

Fig. 4. Comparison of batch data with Model 1 for production of lipid, F.

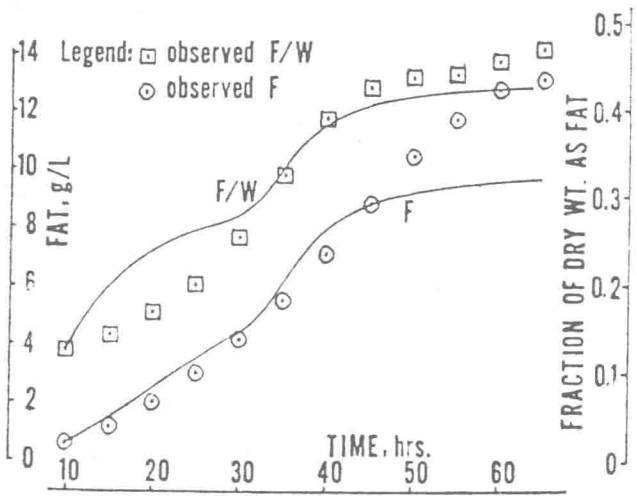

Fig. 5. Comparison of batch data with Model 2 for production of lipid, F.

KINETICS OF BIOFOULING IN SIMULATED WATER DISTRIBUTION SYSTEMS USING CSTR

by

Temkar M. Prakash
Department of Civil Engineering
University of Missouri
Columbia, MO 65211

Introduction

The significance of the problems associated with chemical and biotic degradation of water quality is exemplified by the urgency with which U.S. EPA issued the secondary maximum contaminant levels (SMCL). A detailed literature review presented by Lee (1) indicates that the water quality changes in most distribution systems may be associated with microbial growths within systems. However, it should be emphasized that how much of this problems can be attributed to the microorganisms is still debatable.

Even though drinking water is characterized by extremely low nutrient concentrations, increased number of bacteria is observed in some distribution systems (2). In certain cases, the increases correlate roughly with detention time and with decline in chlorine residual. In waters containing low concentration of organic matter, the microorganisms migrate to the interfaces where interfacial trapping and/or preferential growth takes place. Hence, the apparent deficiency in organic carbon in potable water is probably redressed by the continuous flow velocity at the surface of the pipe (2). As the microorganisms grow on the surface, many of the cells are sloughed off into the medium and are thus measured in the viable counting procedure. The increased biological growth on pipe surface in a distribution system may lead to loss of chlorine residual which is usually related to oxygen depletion due to microbial respiration. In certain areas limitation of oxygen may lead to growth of certain anaerobic bacteria which may produce odor-causing compounds such as sulfides. The iron mains and the subsequent precipitaion of the corrosion products may yield "red water" and increased number of bacteria in the bulk of the water.

Purpose and Scope of Research

The objectives of this investigation were to establish the kinetics of growth of heterotrophic microorganisms as found in water distribution systems and subsequently develop a rational index to measure water quality degradation by attached microbes. A single continuous stirred tank reactor system was used to simulate the attached heterotrophic microbial growth in distribution systems under low shear conditions and develop kinetics of the attached microbes under low substrate levels. Additionally, batch culture experiments were conducted to study the characeteristics of the microbial growth.

Experimental Procedure

In all experiments, biofilm growth on glass slides suspended in single CSTR's was measured. Sterile concentrated substrate was pumped into the reactor and was diluted to a desired concentration by pumping tap (dilution) water into the reactor at a predetermined rate. The tap water was untreated and the microorganisms present in the water served as the microbial inoculum. The feed water was provided with carbon (glucose), nitrogen (ammonium chloride) and phosphorous (potassium phosphate) in the ratio of 25:4:1, respectively. All other nutrients required for the microbial growth were as present in sufficient amounts in the dilution water. Experiments were designed to determine the effect of substrate concentrations and detention time on growth of biofilms.

Biofilm thickness, attached biomass, ATP level in attached biomass, polysaccharides in biofilm, change in TOC, and change in dissolved oxygen were measured as quantitative estimates of biofouling. The biofilm thickness was determined using a microscope (Olympus-model BH) provided with a graduated micrometer. Biomass on glass slides was determined by drying the slides at 100°C. Cold sulfuric acid (0.6 N) was used to extract the ATP from the biofilm. ATP concentration in the extracts was assayed by the luciferin-luciferase assay. Total polysaccharide content of the biofilm was determined using phenol-sulfuric acid colorimetric technique. The TOC concentrations in influent and effluent samples from the CSTR were determined using Beckman Model 915 TOC analyzer.

Batch culture tests were conducted by inoculating tap water supplemented with carbon (glucose), nitrogen (ammonium chloride), and phosphorus (potassium phosphate) in the ratio of 25:4:1, respectively. The inoculum was prepared by spread plating tap water sample on standard plate count media and harvesting the resulting colonies in phosphate buffer. The flasks containing initial concentrations of 800, 400, 200 and 80 mg/l as carbon were incubated at 25°C in a shaker. The microbial growth was measured as Absorbance at 540nm using a spectrometer. At the end of the experiment the biomass and final glucose concentration was measured.

Results and Discussion

Kinetics of Biofilm Growth

Experiments were conducted to determine the kinetics of biofilm growth at various substrate loading rates, 1.2×10^{-3} to 3.6×10^{-3} mg TOC per hour per sq. cm. Experimental substrate consumption rates observed at different substrate loading rates were calculated at dynamic equilibrium. Consumption rates, as shown in Figure 1, is linearly related to substrate loading rate. Assuming that substrate removal depends on viable cell mass, it follows that equilibrium biologic growth or steady state biofilm thickness is also linearly related with equilibrium consumption rate. This relationship is clearly demonstrated in Figure 2. Results from other biofilm research studies (3) indicate that substrate consumption rate increases with increasing biofilm thickness up to some critical thickness, after which removal rate is constant. The biofilm thickness obtained when substrate consumption rate becomes constant is termed "active" thickness and corresponds to the depth of substrate penetration into the biofilm. As seen in Figure 2, the substrate

consumption rate did not attain steady state for the entire range of biofilm thickness studied. Hence, it can be postulated that under the tested substrate loading rates, the entire biofilm remained active, and there was no diffusion limitation.

The rate of biomass production was estimated by measuring specific film growth rate as described by LaMotta (4). The rate of biofilm growth, as measured by biofilm thickness under different substrate loading conditions is shown in Figure 3. In all experiments the biofilm development is observed to follow a typical exponential pattern. The calculated values of the specific growth rate, reported in Table 1, did not show significant variation for the range of substrate loading conditions tested.

Batch Culture Kinetics

Microbial growth characteristics observed in batch culture tests are shown in Figure 4. Pigmentation was observed in all systems after 25 to 30 hours of inocubation. The pigment, greenish yellow, is due to a commonly found microorganism in tap water. Biphasic growth was observed for 800 and 400 mg/l C levels. This was probably due to growth factors that came in with inoculum which gave high initial growth rate and the exhaustion of these resulted in a second lag phase. An alternate mechanism appears to be activated during the lag phase which leads to second growth phase. For 200 and 80 mg/l C concentrations slow but continuous growth was observed probably because the alternate mechanism became operational before the intial supply was exhausted. From the experimental data an average yield of 0.425 mg biomass per mg of glucose consumed was calculated. Apparently, the carbon source (glucose) was the only limiting substrate.

Diffusion of Substrate into Biofilm

To a large extent, diffusivity of nutrients into biofilm controls the characteristics of attached growth systems. Using a porous catalysmodel (5), the effective diffusivities was calculated for the various substrate loading conditions. The observed values of effective diffusivities, shown in Table 1, are in the same range as reported in the literature (5,6). The corresponding maximum depth of penetration of substrate are also presented in Table 1. Comparing the measured steady state biofilm thickness, it is observed that the substrate penetrated the entire film. Apparently, the entire biofilm was active.

Oxygen Consumption in Biofilms

Depletion of oxygen in water distribution systems has been considered as one of the symptoms of water quality degradation (2). Also, a significant portion of the dissolved oxygen (DO) depletion is attributed to microbial respiration. An estimation of oxygen consumption in biofilms, described by two coefficients, oxygen demand for oxidation of substrate and specific rate of oxygen consumption for maintanence metabolism, was calculated by equilibrium material balance as shown in Figure 5. Under the experimental conditions, the biofilm consumed 0.52 mg of oxygen per g cell per hour for maintanance metabolism, and demanded about 0.73 g of oxygen per g of substrate consumed (as TOC) for oxidation and synthesis.

Biofouling Activity Measurement

Several techniques have been proposed in the literature for

monitoring biofouling. Techniques for monitoring bioflim development in water distribution systems include, among others, measurement of frictional resistance in a pipe, biofilm mass, biofilm thickness, protein concentration, polysaccharide concentration etc. However, the above mentioned techniques do not measure the activity of viable microbes which cause biofouling. It is well known that the physiological state and metobolic role of microorganisms are dependent on several factors, such as nutrient content of the water, and environmental conditions such as pH, temperature, and hydrodynamic forces.

In a biofilm, ATP concentration may give an accurate estimate of active biomass. Polysaccharide concentration can also be used as a measure of biofilm activity. However, biomass measurements do not differentiate between the active and dead cells in the biofilm. Based on the proposed study, an index based on the substrate consumption rate and biofilm activity is being prepared.

$$\text{Biofilm Activity Index} = \frac{\text{Substrate Consumption Rate}}{\text{Biofilm Activity}}$$

In the present study the substrate consumption rate was calculated from glucose consumption by the biofilm. The biofilm activity was determined by measuring ATP and polysaccharide concentration of the attached biomass.

Measured ATP and polysaccharide concentrations of the biofilm under various substrate loading conditions is compared with biofilm density in Table 2. Activity indices based on the ATP, polysaccharide concentrations, and mass of the biofilm are presented in Table 3. It is noticed that the index calculated using biomass increases with substrate loading rate, whereas the ATP polysaccharide indices decreased for the highest loading rate (3.6×10^{-3} mg/cm^2-h) compared to that for a rate of 3.0×10^{-3} mg/cm^2-h. This may be due to the decrease in activity of biomass. However, more data are needed to substantiate the use of such an activity index. Nevertheless, use of ATP for the determination and quantitation of physiological and metobolic state of microorganisms present in the biofilm appears promising.

Significance of Study

The onset of water quality of problems in a distribution system is usually brought to light by consumer complaints. However, there is no sound basis either to identify the nature of the problem or to confirm the effectiveness of remedial methods. The results obtained so far in the present research uniquely show that biofouling can occur under extremely low nutrient conditions that prevail in actual water distribution systems. The CSTR system used in this research can be easily adopted to monitor bacterial activity in the problem area of the distribution system. The monitoring system installed and operated with the drinking water in the problem area, and utilzing the methods developed in this research can effectively be used for collecting information on chemical changes broght forth by bacteriological growth. Laboratory experiments are now being conducted to correlate the chemical changes caused by biofouling the ATP index in monitoring systems using cast iron and copper slides. The effect of disinfectants on biofouling activity are also being studied.

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