Statistical Analysis of Growth of Microorganisms

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A mathematical model is derived in the form of a functional equation to describe a population dynamics of microorganisms (bacteria). The individual cells are due to experience a cycle of growth and division during the proliferation period.

The interdivision growth rate of bacterial cells (*Azotobacter vinelandii*) in a continuous and steady state culture is assessed by introducing a probability of division and by using the cell size distributions which are measured with the Coulter counter.

Sigmoid patterns, the single-cell volume versus time, agree incidentally with the previous data which have been published by Harvey et al. from an approach devoid of a particular model. A mutual relationship between the microbial activities from the macroscopic scale of cell mass concentration and those from a statistical analysis is discussed briefly.

Powell (*20*) presented the necessity of differentiating the specific growth rate of bacteria on the mass basis from that rated on the cell number concentration. Painter et al. (*18*) also presented a statistical approach to the generation time distribution $f(\tau)$ of individual cells.

A step further to reveal the growth rate $G(v)$ of bacteria by the statistical means was presented by Collins and Richmond (*7*). This method comprises the observation of cell size distribution in the steady state, and the assessment (or assumption) of both distributions in size of mother cells just prior to their divisions and daughter cells immediately after their birth. Harvey et al. (*11*) determined $G(v)$ of *Escherichia coli* and *Azotobacter agilis* by the procedure of Collins et al.

Another procedure to determine $G(v)$ was suggested recently by Anderson et al. (*4*) and Ramkrishna et al. (*22*). By assuming the rate of cell division and by observing the cell volume distribution in steady state, Anderson et al. determined $G(v)$ (*4*).

Regarding the possibility of bacterial division, Koch and Schaechter (*14*) suggested the critical size, implying that the cells grow exponentially to the critical size either in mass or in length and then divide precisely into halves. The concept which is supported by Powell (*21*) will also be used here to facilitate the statistical analysis.

More recent publications by other workers on the bacterial population dynamics take due consideration of cell age and mass distribution (*5, 6, 8, 16, 19*) and death rate (*24*). Indeed, the bacterial population dynamics has also been the subject of chemical engineering.

Actually, the significance of this statistical approach in the crystallization particle technology as started from the Liouville equation (*12, 23*) has recently been pointed out. Fredrickson and Tsuchiya (*9*) published the equation of the microbial age conservation in a flow reactor.

In fact, the analysis of the population dynamics of bacterial cells resembles the study on crystallization or liquid-liquid dispersion phenomena which are of potential significance in the chemical industry. However, a substantial difference should be noted between the growth and division of the bacterial cells and those observed with the crystallization and/or liquid-liquid dispersion, because the bacterial cell divides, once the intracellular substance such as DNA reaches a specific level, least likely to be affected by the physical factors involved in a flow reactor.

The purpose of this work is to search conversely for the growth rate of individual cells from the measurement of size (volume) distribution of *Azotobacter vinelandii* by assuming the critical volume beyond which each cell should be subjected to the division with a specific probability.

THEORETICAL BACKGROUND

Suppose a reactor vessel be charged continuously with a fresh medium at a constant rate and the medium be discharged from the vessel at the same rate, a working volume of the complete-mixing type of reactor remaining unchanged.

A population balance on the total number of bacterial cells which should comprise those entering and/or leaving a small range between v_x and v_y in individual cell volume in the reactor is considered.

The number of cells which enter into the region comes partly from the interdivision growth of cells smaller than v_x and partly from the division of cells larger than v_y. The "leave" of cells in number from the domain (v_x, v_y) is attributed to the growth of cells, the volumes of which are v_y. The division and flow of cells whose volumes ranging originally from v_x to v_y must also be considered.

Regarding the specific region from v_x to v_y, the population balance for Δt is shown by the following equation:

$$V_R\Delta\left(\int_{v_x}^{v_y} n(t)f(v,t)dv\right) = I_R(v_x,t)\Delta t - I_R(v_y,t)\Delta t - \left(\int_{v_x}^{v_y} J_R(v,t)dv\right)\Delta t + \left(\int_{2v_x}^{2v_y} 2J_R(2v,t)dv\right)\Delta t$$

$$- F_R n(t) \left(\int_{v_x}^{v_y} f(v,t)dv \right) \Delta t \quad (1)$$

where

$$I_R(v,t) = V_R n(t) f(v,t) G(v) \phi(v) \quad (2)$$

$$J_R(v,t)\Delta t = (2^{\Delta t/\theta(v)} - 1)[V_R n(t) f(v,t)(1 - \phi(v))] \quad (3)$$

Here, $J_R(v,t)\Delta t$ implies the increase of cell number for Δt in the flow reactor; especially the term $(2^{\Delta t/\theta(v)} - 1)$ is the net incremental ratio of cell number due to equivolume division. Clearly, the probability $\phi(v)$ of cellular growth without experiencing equivolume division is independent of the time $\theta(v)$ required between the successive divisions.

Individual cells proceed to grow during $\theta(v)$ before they experience another division. It is prohibitive to determine experimentally the value of $\theta(v)$ and the time zero during the $\theta(v)$ time period. Conversely, this statistical means has an advantage of permitting one to assess $\theta(v)$, as will be elaborated later on in this work.

Needless to say, Equations (1) to (3) preclude the discussion of a nonproliferation system; the bacterial growth incorporating necessarily the interdivision growth and the subsequent division in the flow reactor is the principal subject of these equations.

If the interdivision growth is neglected, the physical meaning of $\theta(v)$ is merely the relaxation time between divisions. However, this is rarely possible, because the individual cell volumes become infinitesimally small eventually.

If $(v_x - v_y) \to 0$ and $\Delta t \to 0$, Equation (1) is rearranged with reference to Equations (2) and (3) as follows:

$$\frac{d}{dt}[n(t)f(v,t)] = -n(t)\frac{d}{dv}[f(v,t)G(v)\phi(v)]$$

$$-\frac{\ln 2}{\theta(v)} n(t) f(v,t)[1 - \phi(v)] + 4\frac{\ln 2}{\theta(2v)} n(t) f(2v,t)$$

$$[1 - \phi(2v)] - Dn(t)f(v,t) \quad (4)$$

provided that

$$D = F_R/V_R \quad (5)$$

$$2^{\Delta t/\theta(v)} - 1 \doteq \frac{\ln 2}{\theta(v)}\Delta t$$

Equation (4) represents a general picture of a dynamic population of bacterial cells in a flow reactor.

If the steady state is realized, the left-hand side of Equation (4) becomes zero and the equation reduces to

$$Df(v) = -\frac{d}{dv}[f(v)G(v)\phi(v)]$$

$$-\frac{\ln 2}{\theta(v)} f(v)[1 - \phi(v)] + 4\frac{\ln 2}{\theta(2v)} f(2v)[1 - \phi(2v)] \quad (6)$$

The death rate and cellular age of the bacteria are overlooked in Equation (6) owing to a continuous cultivation of the cells.

Eakman et al. have presented a model on the microbial population dynamics (8). Equation (6) above is essentially different from their model in the sense that the probability $\phi(v)$ and the relaxation time $\theta(v)$ are introduced here. Factor 4 in front of the term involving $f(2v)$ in the right-hand side of Equation (6) is shown erroneously by 2 in the corresponding term of Eakman et al. (8).

The following arrangement is required in order to solve the functional equation [Equation (6)], because unknown functions $f(v)$, $G(v)$, $\theta(v)$, and $\phi(v)$ are in the same equation.

Integrating both sides of Equation (6) with respect to v from $v = 0$ to $v = \infty$, and rearranging, we get

$$D = \ln 2 \int_0^\infty \frac{f(v)[1 - \phi(v)]}{\theta(v)} dv \quad (7)$$

provided that

$$\int_0^\infty f(v)dv = 1, \quad f(0) = 0, \quad f(\infty) = 0,$$

$$G(v) = \text{finite (assumed)}$$

In connection with Equation (7) the following two assumptions are made for ease of further analysis. First, $\theta(v) = \theta =$ constant for each value of D; namely

$$D = \frac{\ln 2}{\theta} \int_0^\infty f(v)[1 - \phi(v)] dv \quad (8)$$

No empirical evidence to support or denounce this assumption seems to have been published.

The second assumption is for the probability function $\phi(v)$ relevant to the bacterial growth free from the division.

$$\phi(v) = \exp[-(v/v_c)^\xi] \quad (9)$$

Equation (9) suggests that the division probability is about 63% irrespective of the value of ξ when the bacteria size reaches the critical volume v_c. The equation also suggests that the division probability becomes even greater with the increase of ξ value, if the cell size exceeds the v_c value. In this analysis the value of $\xi = 2$ was taken a priori. With the increase and/or decrease of ξ taken other than the value of $\xi = 2$, $G(v)$ is affected by the cell volume v more and/or less sharply when v becomes larger than v_c.

Although one can take any value of ξ in this respect, further discussion on the value of ξ will be made with reference to the result of $G(v)$ which appears later on.

Equation (8) can, then, be rewritten as

$$D = \frac{\ln 2}{\theta} \int_0^\infty f(v)[1 - \exp\{-(v/v_c)^2\}] dv \quad (10)$$

Equation (10) shows a relationship between the values of θ and v_c, once the distribution $f(v)dv$ is given for each value of D. If the value of $\bar{v}_c$ which implies the average of critical size of cells independent of D is determined, another average $\bar{\theta}$ is due to be fixed, independent of D.

Supposing that the cell size distribution $f(v)dv$ be observed in the continuous culture with different values of D, and, both values of $\bar{v}_c$ and $\bar{\theta}$ be predetermined properly, an integration of Equation (6) with respect to v from $v = 0$ to $v = v$ presents a solution to $G(v)$ as shown below.

$$G(v) = \frac{-D\int_0^v f(v)dv + \frac{\ln 2}{\bar{\theta}}\left(2\int_0^{v/2} f(v)\,[1 - \exp\{-(v/\bar{v}_c)^2\}]\,dv - \int_0^v f(v)\,[1 - \exp\{-(v/\bar{v}_c)^2\}]\,dv\right)}{f(v)\exp\{-(v/\bar{v}_c)^2\}} \quad (11)$$

The bacterial growth function $G(v)$ can be determined from Equation (11). If the function $G(v)$ $(= dv/dt)$ is assessed, it is easy to demonstrate a relationship between the values of v and t.

$$\int_{v_0}^{v} \frac{dv}{G(v)} = t \qquad (12)$$

EXPERIMENTAL PROCEDURE

Continuous Culture of *Azotobacter vinelandii*

The test organism used in this work was *Azotobacter vinelandii* IAM 1078 (ATCC 9046). The modified Burk's medium (free from nitrogenous component) replacing sucrose with glucose was used. The composition of the culture medium used, the operating conditions of precultivation in shaken flasks, batch culture in a reactor (nominal volume $=$ 29 liters, working volume $\doteqdot$ 13 liters) before starting the continuous culture (chemostat) with the same reactor vessel, and some precaution required to secure the chemostat were exactly the same as those discussed in previous studies (*1, 17*).

The fully baffled reactor (rotation speed of impeller (standard flat-blade turbine) $=$ 750 r.p.m.; aeration rate $=$ 26 liter/min. at N.T.P.) was charged continuously with the fresh medium such that the reciprocal of overall retention time of the flowing medium inside the vessel ranged from 0.10 to 0.25 hr.$^{-1}$ (pH $=$ 7.0 to 7.2, temperature $=$ 30°C.)

The concentration S_0 of glucose in the fresh medium was $S_0 = 5$ mg./ cu. cm. throughout, whereas the residual glucose concentration S_R in the effluent from the reactor was of the order of $S_R = 2$ to 3 μg./cu. cm. (checked by the Glucostat method). Since the dissolved oxygen concentration in the flowing medium inside the reactor measured with the Beckman oxygen probe showed all the time a level of 5 p.p.m., the operation was confirmed to be glucose limited.

Intermittent samplings of the medium from the reactor to measure the cellular concentration X mg./cu. cm. at 610 mμ in wavelength and the residual concentration S_R of glucose were required to justify the chemostat culture in which both values of X and S_R were independent of time t.

Some preliminary tests to determine the size distribution of the bacterial cells by the Coulter counter showed that the size distribution was also independent of time in the chemostat.

Determination of the Cell Size Distribution

The size distribution of *A. vinelandii* sampled from the chemostat culture was measured with the Coulter counter, Model A (*10, 15, 25*).

The cell suspension diluted appropriately was charged into a beaker containing an aqueous solution of 1% sodium chloride (volume $\doteqdot$ 50 cu. cm.). Before preparing the sodium chloride solution, it was subjected to a millipore filter for cleaning to minimize the noise of the electronic counter. The millipore filter used had an average pore size of 0.45 μ. The Coulter counter was calibrated with the standard polystyrene latex spheres ($\bar{d}_p = 1.857$ μ).

RESULTS AND DISCUSSION

Cell Size Distribution

An example of the cell size distribution is shown in Figure 1. The total cell number in each assessment was of the order of 10^4. Solid and open circles in the figure indicate the difference in observation date.

At least two days prior to the measurement of the size distribution, a continuous cultivation of *A. vinelandii* was initiated to establish the chemostat, from which samplings were made. Each chemostat that continued for a couple of days was disassembled, if required.

The size distribution adopted finally was either the data points with open circles or those of solid ones. Apparently the optional adoption of the distribution set of data does not affect appreciably the analysis using a digital computer.

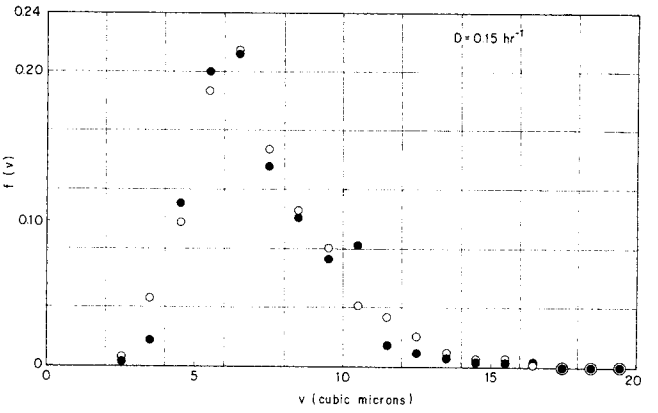

Fig. 1. Size distribution of cells (*Azotobacter binelandii*).

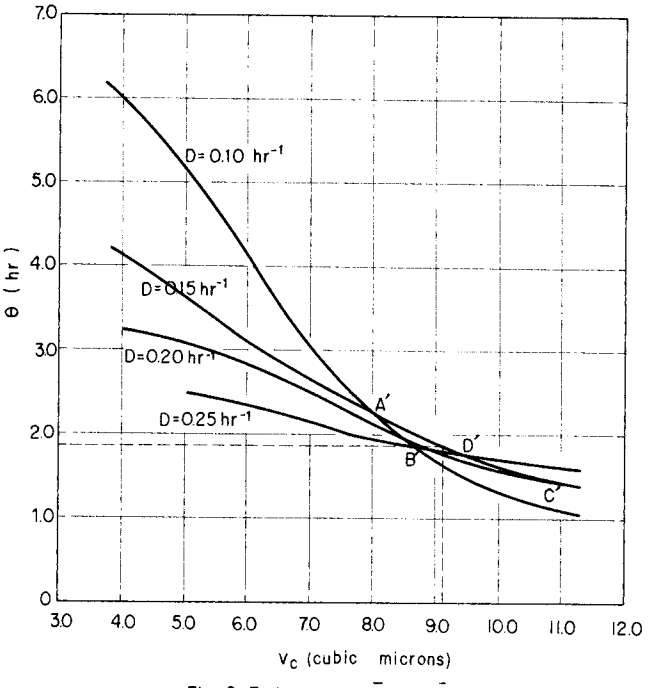

Fig. 2. Estimation of $\bar{v}_c$ and $\bar{\theta}$.

Determination of v_c and θ Values

Equation (10) was used to calculate with a digital computer (HITAC 5020F, Computer Center, University of Tokyo) the relationship between v_c and θ, provided the size distribution $f(v)dv$ for each D was given (cf. Figure 1).

The actual range of calculation for v was from $v \doteqdot 2.5$ to $v \doteqdot 18$ μ^3 (see Figure 1). If the value of $f(v)$ was less than the three orders below the decimal part, it was assumed to be zero.

The result of computation θ versus v_c is shown in Figure 2, parameter being D. Since the value of v_c is assumed here to remain fairly unchanged regardless of D, the value of θ does not differ appreciably depending on the value of D, if the size distribution $f(v)dv$ does not depend remarkably on the value of D as was actually experienced in this work.

Although the intersection of each curve extends as shown in Figure 2 over an area, A', B', C' and D', it is not

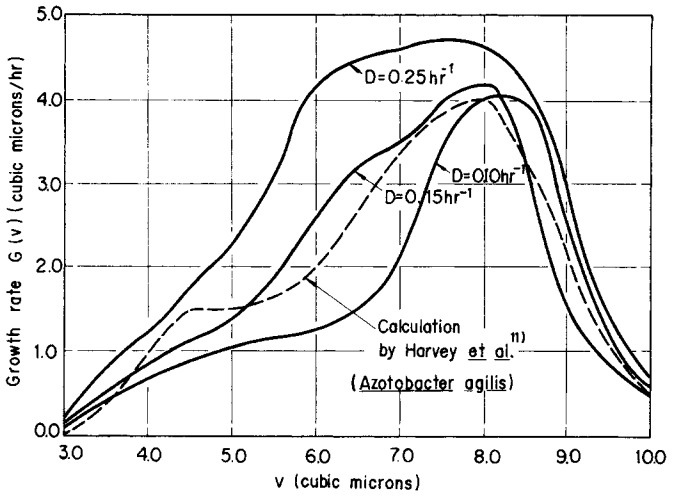

Fig. 3. Growth rate of *Azotobacter vinelandii*.

too prohibitive to take the values of $\overline{\theta}$ and $\overline{v}_c$ as $\overline{\theta} = 1.85$ hr. and $\overline{v}_c = 9.1\ \mu^3$ in this example. If the intersection of each curve scatters too widely or if any of the curves in the figure do not intersect at all, one could change the value of ξ in Equation (9) such that the values of $\overline{v}_c$ and $\overline{\theta}$ could be located without difficulty.

Determination of G(v)

Since the values of $\overline{\theta}$ and $\overline{v}_c$ were determined so far, Equation (11) was used to calculate $G(v)$ with the computer. The result of calculation of $G(v)$ is shown in Figure 3 only for a specific range of v from $v = 3.0$ to $v = 10.0$ μ^3 (see solid curves).

The fact that each curve exhibits some undulated region, especially for v less than 8 μ^3, in the figure might have resulted from the more scattering of the corresponding data for the region of v in the size distribution (Figure 1 and other figures not shown here).

A broken curve in Figure 3 is cited from the work of Harvey et al., who published the growth rate of individual cells (*Azotobacter agilis*) harvested from the logarithmic growth phase in a batch culture (*11*).

Although the procedure for the broken curve in the figure is entirely different from that attempted originally in this work, the fact that these bacterial cells reveal the same pattern of $G(v)$ is still interesting.

Patterns of $G(v)$ for *Escherichia coli* (*11*) and Chinese hamster cells (*4*) were also similar (not shown here) to Figure 3. A claim by other workers that $G(v)$ is exponential or linear with respect to v is not necessarily justified in view of Figure 3.

The fact that $G(v)$ determined thus far by the distinctly different methods is similar points out that the assumptions in this work regarding the value of ξ in Equation (9) and in addition, pertaining to the average values of $\overline{\theta}$ and $\overline{v}_c$ in Equation (10), cannot be totally invalidated.

Referring to Equation (12) the growth rate functions in Figure 3 were integrated using the computer. The value of v_0 at $t = 0$ was taken arbitrarily as 3.75 μ^3 from Figure 3.

The result of computation is shown in Figure 4. An interesting fact, that the interdivision cell growth is sigmoid against time, is noted from the figure. With the increase of D value, the period of time required for the cells to reach the specific value of $\overline{v}_c$ ($= 9.1\ \mu^3$) shortens clearly. For solid curves in Figure 4 the growth of v beyond the critical value of $\overline{v}_c$ originates from the assumption that the cells larger than $\overline{v}_c$ still have the probability ($= 37\%$) of growth without proceeding to the division.

Specific Growth Rate, μ as Viewed from the Cell Size Distribution

Assuming that the individual cell density ρ is independent of the cell volume v (*3*), the cell mass concentration X is expressed by the following equation:

$$X(t) = \rho(t) \int_0^\infty n(t) f(v,t) v\, dv \qquad (13)$$

Differentiating both sides of Equation (13) with time t

$$\frac{dX(t)}{dt} = \rho(t) \int_0^\infty \frac{d[n(t)f(v,t)]}{dt} v dv + \frac{d\rho}{dt} \int_0^\infty n(t) f(v,t) v dv$$

If Equation (4) is substituted into the integrand of the first term on the right-hand side of the above equation

$$\frac{dX(t)}{dt} = \rho(t) n(t) \int_0^\infty f(v,t) G(v) \phi(v) dv - D\rho(t) n(t) \overline{v} + \frac{d\rho}{dt} n(t) \overline{v} \qquad (14)$$

Rearranging Equation (14)

$$\frac{1}{X}\frac{dX}{dt} = \frac{1}{\overline{v}} \int_0^\infty f(v,t) G(v) \phi(v) dv - D + \frac{1}{\rho}\frac{d\rho}{dt} \qquad (15)$$

In a nonflow system (batch culture, $D = 0$), Equation (15) reduces to

$$\frac{1}{X}\frac{dX}{dt} (\equiv \mu_x) = \frac{1}{\overline{v}} \int_0^\infty f(v,t) G(v) \phi(v) dv + \frac{1}{\rho}\frac{d\rho}{dt} \qquad (16)$$

The left-hand side of Equation (15) becomes zero in the steady state of the flow reactor. In this case the reciprocal of the retention time of the flowing medium D in the

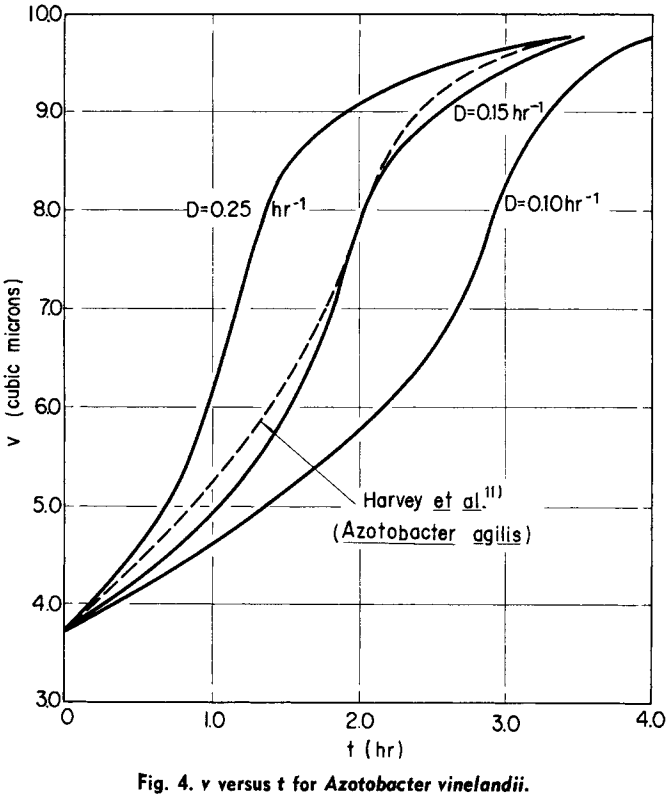

Fig. 4. v versus t for *Azotobacter vinelandii*.

reactor must be equal to the specific growth rate μ_x of the cells in view of the cellular mass balance. Then, from Equation (15)

$$D = \frac{1}{\overline{v}} \int_0^\infty f(v)G(v)\phi(v) + \frac{1}{\rho}\frac{d\rho}{dt} = \mu_x \qquad (17)$$

The optical measurement of cell growth in terms of UOD (units of optical density) disregards the second term on the right-hand side of Equation (16) or (17). Then

$$\mu_x = \frac{1}{\overline{v}} \int_0^\infty f(v,t)G(v)\phi(v)dv \qquad (18)$$

The specific rate of change μ_n in cell number concentration in the nonflow system ($D = 0$) is defined from Equation (4) by integrating both sides of the equation from $v = 0$ to $v = \infty$.

$$\mu_n \equiv \frac{1}{n(t)}\frac{dn(t)}{dt} = \frac{\ln 2}{\theta} \int_0^\infty f(v,t)\,[1 - \phi(v)]\,dv \qquad (19)$$

provided that θ = constant, as was assumed earlier.

Clearly, the implications of μ_x and μ_n are different [cf. Equations (18) and (19)]. It is natural to find out the disparity between the plot of UOD against time and that of cell number concentration against time on a semilogarithmic paper when the specific growth rate of a bacterium is under examination. Although this inconsistency between μ_x and μ_n has long been advocated empirically (*13, 20*), Equations (18) and (19) indicate the discrepancy, if any, in more concrete terms.

For the steady state in the flow reactor, it is evident from the cellular mass and/or number balance that

$$\mu_n = D = \mu_x \qquad (20)$$

CONCLUSION

1. A functional equation for population dynamics of microorganisms (bacteria) in a flow reactor has been derived to show the relationship between the bacterial interdivision growth rate and the size distribution of the cells.

2. Assuming the division probability in conjunction with the concept of critical cell size relevant to the division, the determination of the growth rate has been illustrated with special reference to the experimental data on the size distribution of a bacterium (*A. vinelandii*) in a continuous and steady state culture.

3. Different implications of the specific growth rates based on cell number and mass concentrations have been demonstrated.

NOTATION

$\overline{d_p}$ = mean diameter of polystyrene particle, μ
D = reciprocal of retention time of flowing medium inside reactor = dilution rate = F_R/V_R, hr.$^{-1}$
$f(v)$ = cell volume distribution in steady state
$f(v,t)$ = cell volume distribution in unsteady state
F_R = feed rate, cu. m./hr.
$G(v)$ = growth rate of single cells, μ^3/hr.
$I_R(v,t) = V_R n(t) f(v,t) G(v)\phi(v)$
$J_R(v,t)\Delta t = (2^{\Delta t/\theta(v)} - 1)V_R n(t) f(v,t)[1 - \phi(v)]$
$n(t)$ = cell number concentration in unsteady state, 1/cu. m.
S_0 = glucose concentration in fresh medium, mg./cu. cm.
S_R = residual glucose concentration, mg./cu.cm.
t = time, hr.
v_0 = cell volume at $t = 0$ or v value at which $f(v)$ can be assumed to be zero or value of v at which $G(v_0)$ corresponds to $t = 0$
v_c = critical cell volume, μ^3
$\overline{v_c}$ = mean value of v_c
v = cell volume, μ^3
$\overline{v}$ = mean value of v, $\int_0^\infty f(v,t)v\,dv$
v_x, v_y = cell volumes
V_R = working volume of reactor vessel, cu. m.
X = cell mass concentration, mg./cu. cm.

Greek Letters

μ_n = specific rate of change in cell number concentration, hr.$^{-1}$
μ_x = specific rate of change of cell mass concentration, hr.$^{-1}$
θ = time elapsed between initiation of division of mother cell and completion of emergence of two daughter cells, hr.
$\overline{\theta}$ = mean value of θ, hr.
ξ = empirical exponent
ρ = cell density, g./cu. cm.
τ = generation time, hr.
$\phi(v)$ = probability that single cells (volume = v) proceed to grow without experiencing division

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