MICROORGANISMS PRODUCTION

Theoretical Considerations of a Continuous Culture System

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Continuous culture was studied from a theoretical standpoint. Calculations are presented which are useful in interpreting results obtained by this process and in predicting operating conditions. There is a distinct advantage in using a second culture vessel and possibly a third vessel in series to achieve a higher concentration of the product at some fixed culture medium rate. As with batch cultures, sterility requirements of the medium are important, and contaminants must be avoided in operation of the system. Selective growth of variants will disrupt the system.

CONTINUOUS CULTURE can be defined as the cultivation of microorganisms in a system where sterile medium, or a culture of low cell concentration, is added continuously to a culture vessel while product having a higher concentration is removed at the same rate, leaving no net change in volume of culture in the vessel. Compared with a batch process, several advantages are apparent from a production standpoint.

1. A steady load on the utility sources is possible, with no peak loads.
2. A continuous process is more adaptable to automatic control.
3. A uniform product is obtained.
4. Increased production for a given culture vessel is realized.

The feasibility of continuous culture, or continuous fermentation, is demonstrated by its use in many fermentation industries. De Becze and Rosenblatt (*3*) reviewed several major continuous fermentation processes, while Unger *et al.* (*7*) and Bilford *et al.* (*2*) described operational characteristics of two particular continuous processes. On a smaller scale, Gerhardt (*4*) and Moyer (*6*) pointed out ways of studying characteristics of bacteria and some limitations of the continuous method. A theoretical approach to problems which can be studied by the continuous culture technique is given by Monod (*5*).

The purpose of this paper is to develop calculations which will be useful in interpreting results and predicting operating conditions for a continuous culture system. The study will consider three problems:

1. Growth of the desired organism,
2. Replacement of desired organism by genetic variants, and
3. Replacement of desired organism by contaminating organisms.

Growth of the Desired Organism

A schematic representation of a continuous culture system is given in Figure 1. It is believed that only two cases need be considered: (a) sterile medium flowing into a vessel containing organisms (vessel A), and (b) culture flowing into a vessel having an overflow spout (vessel B).

For this discussion, it is envisaged that vessel A will be filled to the overflow spout with liquid having a_0 number of organisms, and vessel B will be empty when operation is begun. A hypothetical growth curve for the organisms under consideration is given in Figure 2. The curve shown applies to the system in which R (the rate of medium flow into vessel A) = 0—a batch system. It is assumed throughout this paper that the culture in each vessel is homogeneous at all times.

Sterile Medium Flowing into Vessel A

In considering sterile medium flowing into a vessel containing organisms, let da/dt represent the instantaneous change in the total number of organisms at time t. Then

$$\frac{da}{dt} = \left[\frac{da}{dt}\right]_{\text{into vessel } A} + \left[\frac{da}{dt}\right]_{\text{growth}} - \left[\frac{da}{dt}\right]_{\text{exit vessel } A} \quad (1)$$

Since sterile medium is to be added to vessel A,

$$\left[\frac{da}{dt}\right]_{\text{into vessel } A} = 0 \quad (2)$$

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The general equation for growth is

$$\left[\frac{da}{dt}\right]_{\text{growth}} = ka \qquad (3)$$

where k is the growth rate constant of the organisms in vessel A. Furthermore, the loss of organisms from vessel A by overflow may be represented as

$$\left[\frac{da}{dt}\right]_{\text{exit vessel } A} = \left[\frac{R}{V_A}\right] a \qquad (4)$$

By substitution

$$\frac{da}{dt} = 0 + ka - \left[\frac{R}{V_A}\right] a \qquad (5)$$

At time t = 0, let $a = a_o$.
Then

$$\ln \frac{a}{a_o} = \left[k - \frac{R}{V_A}\right] t \qquad (6)$$

It is apparent that steady state conditions—i.e., a is constant—can be maintained only if $k - \frac{R}{V_A} = 0$ and under this condition $a = a_o$. If $k - \frac{R}{V_A} < 0$, a will approach 0 at a rate depending on the quantitative negative difference of $k - \frac{R}{V_A}$. If $k - \frac{R}{V_A} > 0$, a will approach ∞ at a rate depending on the quantitative positive difference of $k - \frac{R}{V_A}$.

Since it is expected that in continuous operation steady state conditions would be desired, consider the case where $k - \frac{R}{V_A} = 0$ or $k = \frac{R}{V_A}$—i.e., the growth rate is equal to the dilution rate. If the volume of the vessel, V_A, is fixed, there is only one rate of medium flow, R, at which steady state conditions will be maintained. Consequently, it is advantageous to determine the value of k. Integration of Equation 3 yields:

$$a = a_o e^{kt} \qquad (7)$$

The multiplication of organisms by binary fission also can be represented by the equation

$$a = a_o 2^{\frac{t}{g}} \qquad (8)$$

where g is the generation time of the organism under consideration.

The two growth equations are identical when $k = \frac{\ln 2}{g}$. Hence at steady state

$$\frac{R}{V_A} = \frac{\ln 2}{g} \qquad (9)$$

or

$$R = \frac{\ln 2}{g} V_A \qquad (10)$$

The medium rate, R, at equilibrium as a function of generation time, g, for several effective volumes, V_A, is shown in Figure 3.

Culture Flowing Into Vessel *B* Consideration of culture flowing into a vessel having an overflow spout will be separated into two phases. Phase I extends over the filling of vessel B. Phase II begins when culture starts to overflow from vessel B.

Filling of Vessel *B* (Phase I).

$$\frac{db}{dt} = \left[\frac{db}{dt}\right]_{\text{into vessel } B} + \left[\frac{db}{dt}\right]_{\text{growth}} \qquad (11)$$

$$\left[\frac{db}{dt}\right]_{\text{into vessel } B} = \left[\frac{R}{V_A}\right] a \qquad (12)$$

Under steady state conditions

$$\left[\frac{db}{dt}\right]_{\text{into vessel } B} = \left[\frac{R}{V_A}\right] a_o \qquad (13)$$

Also

$$\left[\frac{db}{dt}\right]_{\text{growth}} = k'b \qquad (14)$$

where k' is the growth constant of the organisms in vessel B.
By substitution

$$\frac{db}{dt} = \left[\frac{R}{V_A}\right] a_o + k'b \qquad (15)$$

Taking $b = 0$ at $t = 0$

$$\frac{1}{k'} \ln \frac{k'b + \left[\frac{R}{V_A}\right] a_o}{\left[\frac{R}{V_A}\right] a_o} = t \qquad (16)$$

The instant that culture begins to overflow from vessel B is at $t = \frac{V_B}{R}$ which is the time necessary to fill the vessel. Substituting this value of t in Equation 16 at overflow

$$\frac{1}{k'} \ln \frac{k'b + \left[\frac{R}{V_A}\right] a_o}{\left[\frac{R}{V_A}\right] a_o} = \frac{V_B}{R} \qquad (17)$$

or

$$k'b = \left[\frac{R}{V_A}\right] a_o e^{\left[k'\left(\frac{V_B}{R}\right)\right]} - \left[\frac{R}{V_A}\right] a_o \qquad (18)$$

At steady state $\frac{R}{V_A} = k$

Therefore

$$k'b = k\, a_o e^{\left[k'\left(\frac{V_B}{R}\right)\right]} - k\, a_o \qquad (19)$$

If $V_A = V_B$, then $\frac{V_B}{R} = \frac{V_A}{R} = \frac{1}{k}$

and

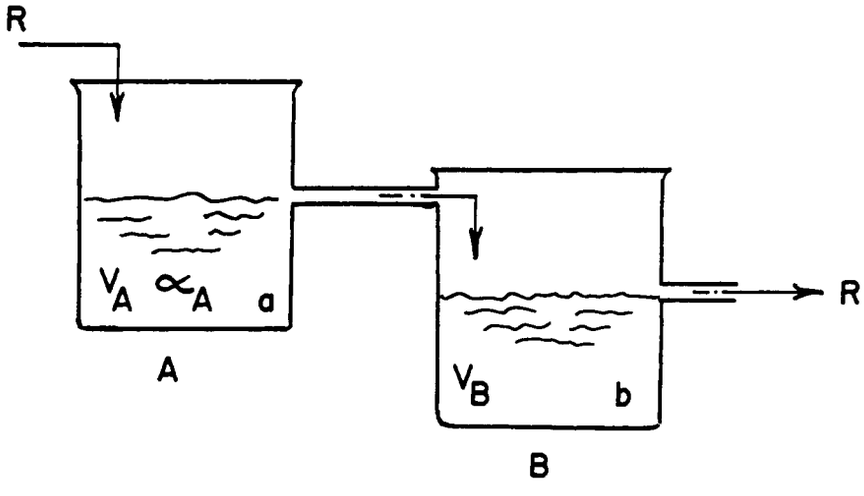
$$b = \frac{k}{k'} a_o \left[e^{\left(\frac{k'}{k}\right)} - 1\right] \qquad (20)$$

During growth, the maximum value of b, the total number of organisms in vessel B, would occur when its growth rate constant $k' = k$, the growth rate constant of the organisms in vessel A. It should be observed that in normal operation k' cannot exceed k, and both will be equal only when the organisms in both vessels are reproducing at the logarithmic growth rate (see Figure 2). For this condition

$$b = a_o [e - 1] = 1.718\, a_o \qquad (21)$$

It is seen that if both vessels have the same volume, then the maximum increase in concentration that could be attained by filling of the second vessel is 1.718 times the concentration in vessel A. If V_A is not equal to V_B, then Equation 19 can be used to predict the number of organisms in vessel B when the vessel is filled to overflowing.

Figure 1. Schematic representation of continuous culture system



Overflow from Vessel B (Phase II).

$$\frac{db}{dt} = \left[\frac{db}{dt}\right]_{\text{into vessel } B} + \left[\frac{db}{dt}\right]_{\text{growth}} - \left[\frac{db}{dt}\right]_{\text{exit vessel } B} \quad (22)$$

$$\left[\frac{db}{dt}\right]_{\text{exit vessel } B} = \left[\frac{R}{V_B}\right] b \quad (23)$$

$\left[\frac{db}{dt}\right]_{\text{into vessel } B}$ and $\left[\frac{db}{dt}\right]_{\text{growth}}$

are the same as in Phase I.

Therefore, with steady state conditions in vessel A

$$\frac{db}{dt} = \left[\frac{R}{V_A}\right] a_o + k'b - \left[\frac{R}{V_B}\right] b \quad (24)$$

Taking b_o as the number of organisms at $t = 0$

$$\frac{1}{k' - \frac{R}{V_B}} \ln \left[\frac{b\left[k' - \frac{R}{V_B}\right] + \left[\frac{R}{V_A}\right] a_o}{b_o\left[k' - \frac{R}{V_B}\right] + \left[\frac{R}{V_A}\right] a_o} \right] = t \quad (25)$$

Transposing

$$\frac{b\left[k' - \frac{R}{V_B}\right] + \left[\frac{R}{V_A}\right] a_o}{b_o\left[k' - \frac{R}{V_B}\right] + \left[\frac{R}{V_A}\right] a_o} = e^{\left[k' - \frac{R}{V_B}\right] t} \quad (26)$$

From the above equation, it is seen that when

$$\left[k' - \frac{R}{V_B}\right] > 0$$

$b \to \infty$ as $t \to \infty$

and when

$$\left[k' - \frac{R}{V_B}\right] = 0$$

b is indeterminate

However, for the latter condition, a solution of Equation 24, for the case $k' = \frac{R}{V_B}$ yields

$$b = \left[\frac{R}{V_A}\right] a_o t + b_o \quad (27)$$

The development of Equation 24 assumes that steady state conditions exist in vessel A so that

$$\left[\frac{R}{V_A}\right] a_o = k a_o \quad (28)$$

Substituting in Equation 27

$$b = k a_o t + b_o \quad (29)$$

Hence $b \to \infty$ with increasing time.

Also, from Equation 26, it is seen that when

$$\left[k' - \frac{R}{V_B}\right] < 0$$

then

$$\lim_{t \to \infty} e^{\left[k' - \frac{R}{V_B}\right] t} = 0 \quad (30)$$

and

$$\lim_{t \to \infty} b = \frac{-\frac{R}{V_B} a_o}{\left[k' - \frac{R}{V_B}\right]} \quad (31)$$

The results given for the conditions of $k' - \frac{R}{V_B} > 0$ and $k' - \frac{R}{V_B} = 0$ show that $b \to \infty$ as $t \to \infty$. This means that if the growth rate constant k' is equal to or greater than the dilution rate, the number of organisms, b, will increase indefinitely with increasing time. However, it is known that there is a maximum number of organisms which one can grow in a given medium. The discrepancy between the theoretical and the known lies in the fact that k' and k are constants only for a given concentration range, above which they decrease to zero. To be able to predict maximum and minimum values of b, one must know the relationship of b as a function of k'. That a stationary value will be realized appears obvious, since as b increases over a certain value, k' will decrease and $k' - \frac{R}{V_B}$ also will decrease, which in turn will decrease b.

If the culture volumes for both vessels are equal, and conditions are such that steady state is realized in vessel A, then $\frac{R}{V_B} = \frac{R}{V_A} = k$. Since, as explained previously, k' can equal but never exceed k in normal operation, then only Equations 29 and 31 apply. k' might be expected to be less than k so that Equation 31 would be most useful when the culture volumes of both vessels are equal. For certain continuous culture processes, it may prove advisable to have the culture volume in vessel B greater than that of the preceding vessel.

The growth rate constant, k, is dependent not only on the organism in process of culture but also on concentration of the organism, medium composition, aeration rates, and temperature conditions. This factor should be determined experimentally for the organism to be used under the processing conditions that will be employed. The variation of k with time for a hypothetical growth curve is shown in Figure 2, and possible concentrations in both vessels at steady state are indicated.

Equation 6 predicts no maximum value for the number of organisms which can be attained. By suitable adjustment of the medium flow, and keeping the volume, V_A, constant, any concentration can be attained within the growth limits of the organism. However, in operation, one is ultimately concerned with the number of organisms which leave the vessel per unit time $\left[\left(\frac{R}{V}\right) a\right]$ and the concentration of the organisms, $\left[\frac{a}{V}\right]$. Most rapid production of organisms occurs when k, the growth rate constant, is at a maximum which is usually, if not always, realized in the logarithmic growth phase.

For many bacteria, the logarithmic growth phase ends at a concentration considerably below the maximum concentration attainable. From the end of the logarithmic growth phase to the maximum stationary growth phase, the growth rate constant, k, decreases steadily to zero. Hence with one vessel, as A, the decision which must be made is whether the effluent should contain a large number of organisms at a low concentration (large volume of product) or a lesser number of organisms at a

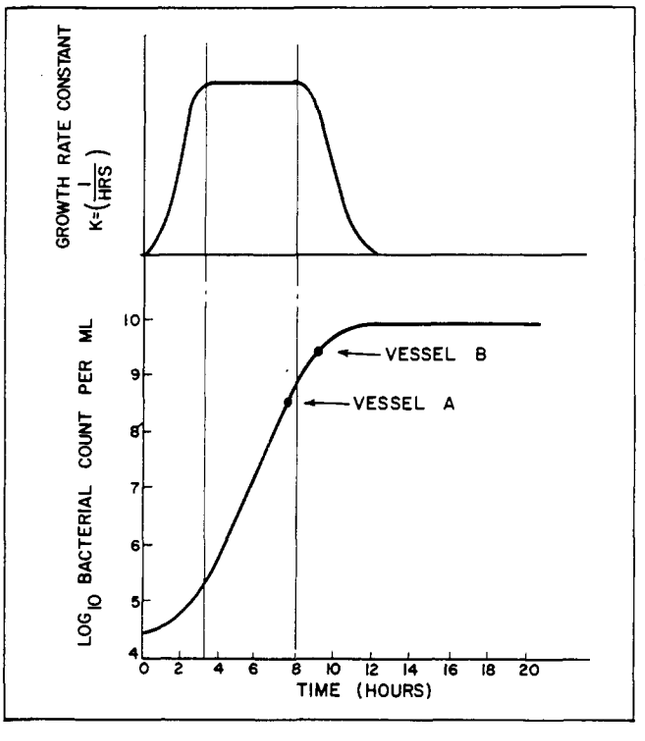


Figure 2. Hypothetical growth curve for *Escherichia coli*

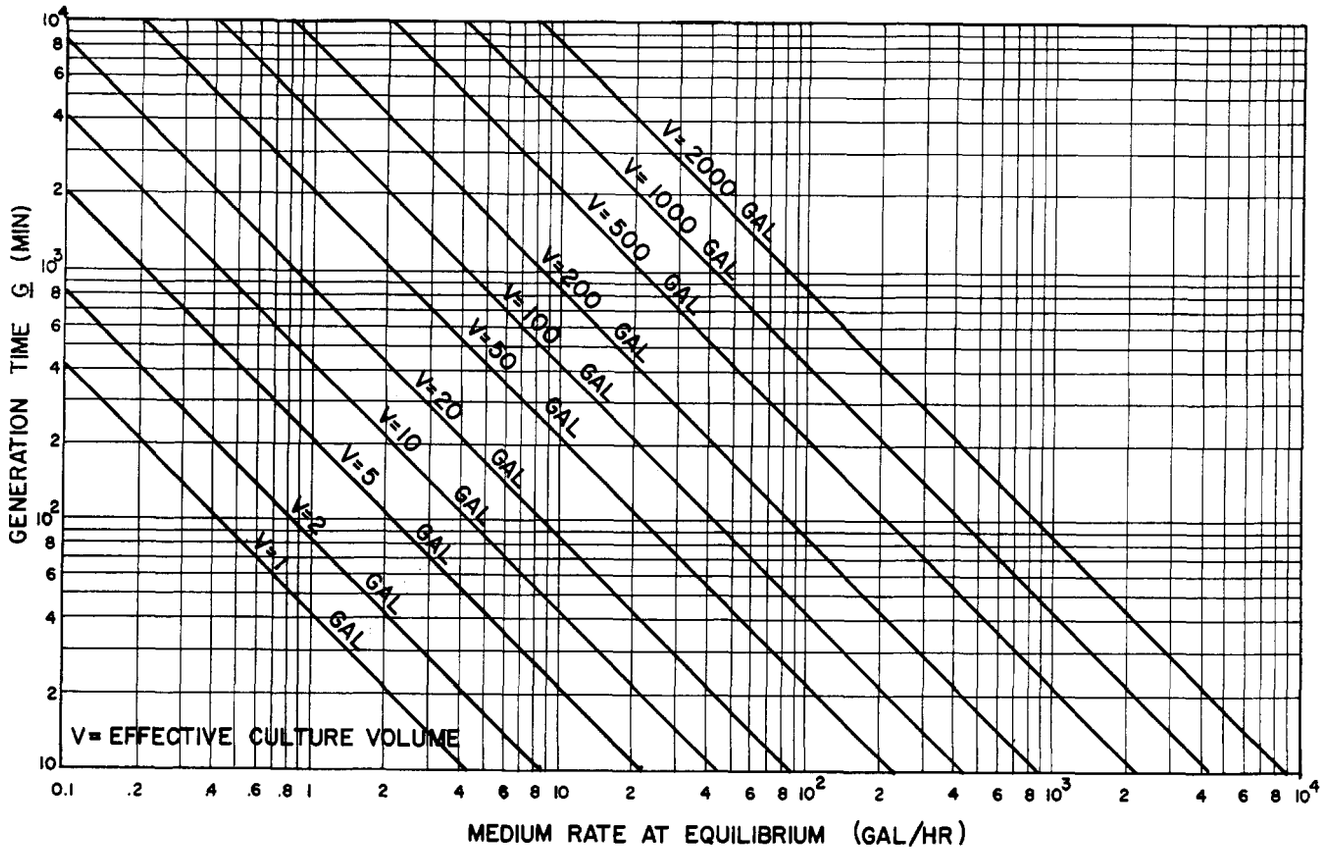


Figure 3. Generation time versus medium rate at equilibrium

higher concentration (small volume of product). Usually, a combination of a large number of organisms at a high concentration would be the most desirable. When the combination cannot be achieved with one vessel, additional vessels can give the desired result.

The theoretical development of vessel B, Equations 11 to 31, shows that for two vessels of equal volume, the rate that would be considered optimum for vessel A and still keep vessel A at steady state will also give steady state conditions in vessel B at a higher concentration. Succeeding vessels would be expected to show additional smaller increases in concentration of the effluent at some fixed rate.

The limit as to the number of vessels to be used should be determined by the growth characteristics of the organism in question. Also, economic considerations would be of prime importance, since it is readily realized that a point will be reached where the cost of adding another vessel would outweigh the advantage of a small increase in concentration of the product.

Replacement of Desired Organism By Genetic Variants

A continuous culture system would be capable of operating indefinitely provided variants or contaminants did not occur in concentrations great enough to cause an unsatisfactory product. Contamination of a culture could be prevented by the proper design, construction, and operation of the production system. However, introduction of genetic variants into the culture cannot be prevented, since genetic variation or mutation occurs randomly as a function of growth.

The following analysis was made considering the case where sterile medium is flowing into a vessel (vessel A), when

1. Steady state conditions exist in vessel A—i.e., the dilution rate is equal to the growth rate of the parent organism, $\frac{R}{V_A} = k$.
2. Increase in the number of variants is due to (a) mutations of the desired or parent organism, and (b) growth of the variants after their origin.

The general equation for the instantaneous rate of change of number of variants may be expressed as

$$\frac{d\alpha_A}{dt} = \left[\frac{d\alpha_A}{dt}\right]_{\text{into vessel } A} + \left[\frac{d\alpha_A}{dt}\right]_{\text{from mutation}} + \left[\frac{d\alpha_A}{dt}\right]_{\text{growth}} - \left[\frac{d\alpha_A}{dt}\right]_{\text{exit vessel } A} \quad (32)$$

For the conditions with sterile medium entering the vessel

$$\left[\frac{d\alpha_A}{dt}\right]_{\text{into vessel } A} = 0 \quad (33)$$

If ϕ is taken as the proportionality factor of mutation, then

$$\left[\frac{d\alpha_A}{dt}\right]_{\text{from mutation}} = \left[\left[\frac{R}{V_A}\right] a_o\right]\phi = ka_o\phi \quad (34)$$

since

$$\left[\left[\frac{R}{V_A}\right] a_o\right]$$

is the instantaneous rate of production of organisms when steady state conditions exist in vessel A.

$$\left[\frac{d\alpha_A}{dt}\right]_{\text{growth}} = k_\alpha \alpha_A \quad (35)$$

$$\left[\frac{d\alpha_A}{dt}\right]_{\text{exit vessel } A} = \left[\frac{R}{V_A}\right] \alpha_A = k\alpha_A \quad (36)$$

By substitution in Equation 32

$$\frac{d\alpha_A}{dt} = 0 + ka_o\phi + k_\alpha\alpha_A - k\alpha_A \quad (37)$$

Let

$$\alpha_A = \alpha_{A_o} \text{ at } t = 0$$

By integration

$$\frac{\alpha_A\,[k_\alpha - k] + ka_o\phi}{\alpha_{A_o}\,[k_\alpha - k] + k\,a_o\phi} = e^{[k_\alpha - k]t} \quad (38)$$

It is seen that when

$$[k_\alpha - k] > 0$$

$$\alpha_A \to \infty \text{ as } t \to \infty$$

and when

$$[k_\alpha - k] = 0$$

α_A is indeterminate.

However, for the condition of $k_\alpha = k$, Equation 37 yields

$$\alpha_A = ka_o\phi t + \alpha_{A_o} = k_\alpha a_o \phi t + \alpha_{A_o} \quad (39)$$

Also, from Equation 38, it is seen that when

$$[k_\alpha - k] < 0$$

then

$$\lim_{t \to \infty} \alpha_A = \frac{-ka_o\phi}{[k_\alpha - k]} \quad (40)$$

The limitations regarding the maximum value of α_A are identical with those considered for the value of b in the previous section.

One important conclusion can be drawn: Regardless of the value of k_α, the product cannot be entirely free of variant organisms, and if k_α is greater than k, a steadily increasing amount of variants will be encountered. For the case where $k_\alpha = k$, α_A probably would increase very slowly, since ϕ, the proportionality factor of mutation, might be expected to be low. Although it is not possible to state with authority an average mutation rate, such a rate probably lies between 1 per 1,000,000 to 1 per 100,000,000 gene duplications.

If selective growth in favor of the undesired type should occur—i.e., $k_\alpha > k$—the system will be thrown out of balance, and operation will have to be stopped. The only condition that will permit uninterrupted operation requires that the growth rate constant of the variant be less than that of the parent organism ($k_\alpha < k$). The minimum number of variant organisms in the culture under these conditions, knowing the growth rate constants, can be predicted from Equation 40.

The time limit in operation, for the conditions where $k_\alpha = k$ and $k_\alpha > k$, is dependent on the tolerance of variant organisms in the product, which is directly related to the proportionality factor of mutation.

This analysis has not been extended to additional vessels, as the addition of more vessels would not alleviate the effect of undesirable variants developing in the first vessel.

Replacement of Desired Organism By Contaminating Organisms

Contamination is one of the most prevalent difficulties encountered in the growth of pure cultures. The theoretical behavior of contaminants in a continuous culture system is considered below. Some simplifying assumptions are made.

1. The medium entering the first vessel, vessel A, is the only point of contamination, and this medium contains X_o number of contaminant organisms per volume V_A.
2. Vessel A is operating under steady state conditions—i.e., $\frac{R}{V_A} = k$.

For these conditions, the situation is similar to that found in vessel B described under Overflow from Vessel B (Phase II).

$$\frac{dX}{dt} = \left[\frac{R}{V_A}\right] X_o + k_x X - \left[\frac{R}{V_A}\right] X \quad (41)$$

where k_x is the growth rate constant of the contaminant X.

Substituting $\frac{R}{V_A} = k$, let $X = X_1$ at $t = 0$

By integration

$$\frac{X[k_x - k] + kX_o}{X_1[k_x - k] + kX_o} = e^{[k_x - k]t} \quad (42)$$

when

$$[k_x - k] > 0$$

$$X \to \infty \text{ as } t \to \infty$$

and when

$$[k_x - k] = 0$$

X is indeterminate.

However, from Equation 41, for $k_x = k$

$$X = kX_o t + X_1 = k_x X_o t + X_1 \quad (43)$$

which indicates as $t \to \infty$, $X \to \infty$

Referring to Equation 42 when

$$[k_x - k] < 0;$$

then

$$\lim_{t \to \infty} X = \frac{-kX_o}{[k_x - k]} \quad (44)$$

If k_x, the growth rate constant of a particular contaminant, is greater than k, the growth rate constant of the desired organism, there can be serious complications from that contaminating organism. If k_x is less than k, then X, the number of contaminating organisms per volume V_A, will approach a constant value which can be predicted from Equation 44. The condition of selective growth is manifested when $k_x > k$.

Conclusions

Continuous culture is an important method of producing microorganisms. This method has many inherent advantages over batch processes. The preceding theoretical treatment, aimed at giving an insight to the mechanism of the process, offers a way of evaluating data from a continuous process, which may aid in the design of equipment.

Steady state conditions can be predicted for a vessel in a continuous culture system where sterile medium flows into a vessel containing organisms, if the growth rate constant of the organism is known.

There is a distinct advantage in using a second vessel and possibly a third vessel, in which steady state will be reached at a concentration which can be predicted only if the growth rate constant as a function of concentration of the organism is known.

Variants should not offer any serious problems if the growth constant of the variant organism is less than that of the desired organism.

Sterility requirements of the medium are important, and contaminants should be avoided in operation of the system.

Selective growth of either variants or contaminants will disrupt the system.

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Nomenclature

- a = total number of organisms in vessel A at time t.
- b = total number of organisms in vessel B at time t.
- g = generation time, hours.
- k, k', k_α, k_x = growth rate constants, $\left[\frac{1}{\text{hour}}\right]$.
- R = rate of medium flow into vessel A, gal./hour.
- $\frac{R}{V}$ = dilution rate $\left[\frac{1}{\text{hour}}\right]$.
- t = time, hours.
- V_A, V_B = volume of culture in vessels A and B, respectively, to the overflow spout, gal.
- X = total number of contaminant organisms.
- α_A = total number of variant or mutant organisms in vessel A.
- ϕ = proportionality factor of mutation—i.e., one mutant is formed per given number of cell divisions.

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