FERMENTATION PROCESS CONTROL

Population Dynamics of a Continuous Propagator for Microorganisms

R. K. FINN and R. E. WILSON[1]
University of Illinois, Urbana, Ill.

As continuous fermentation offers economic advantages over the usual batchwise process, the present work was undertaken to provide a better understanding of the characteristics of a continuous propagator. Laboratory apparatus was developed which allowed sterile propagation of aerobic microorganisms in a stirred-tank reactor of the overflow type. Using *Ps. fluorescens, B. linens,* and a strain of *S. carlsbergensis* a theoretical relation was shown between the mean retention time of the cells in the propagator and their growth rate. By proper adjustment of the flow rates, steady populations were attained. In incompletely buffered media, a steady cycling in yeast population was observed, which was traced to steady fluctuations in the pH which were 90 degrees out of phase with fluctuations in population. The phenomenon appears to arise from the inherent feedback in the system coupled with a metabolic lag. Fermentation of the medium was not complete, as only the propagation of cells was of interest. In a practical process additional holding tanks would be provided, so that end products could be obtained in high yield.

MOST STUDIES OF CONTINUOUS FERMENTATION have been concerned with producing consistently high yields of alcohol, yeast, or other specific materials (*3, 17, 19*). It is appropriate that academic research in the field of bioengineering should undergird these applied studies with a more detailed inquiry into the behavior of living cells held in continuous culture. The purpose of this paper is to begin such an inquiry.

When fermentation is carried out in a cascade of two or more stirred tanks connected in series, the first tank constitutes a propagator. Fresh nutrient is added to the propagator at constant rate and a constant level of liquid is maintained in the propagator by arranging an overflow at the desired height. The rate of withdrawal is at all times equal to the feed rate and is generally so rapid that fermentation within the propagator is not complete, and all nutrients remain in excess of the cell requirements. Under such conditions, the cell population is limited solely by the washout of cells.

Novick and Szilard (*14, 15*) utilized a continuous propagator to study microbial genetics, especially mutation rates. Their apparatus, which they chose to call a chemostat, was operated in such a manner that growth was limited by deficiency of a particular nutrient, rather than by the washout rate.

The present work follows more closely that of Adams and Hungate (*1*). Using a continuous yeast propagator these investigators showed how the flow rate could be predicted from the growth curve of the organism. Several points raised in their paper, however, seemed to warrant further study. In the first place, Adams and Hungate did not observe any appreciable constant-rate phase of growth for yeast in their media. With organisms that show logarithmic growth, the estimation of flow rates could be simplified.

Furthermore, steady populations were not always obtained in the yeast propagator. Although Adams and Hungate (*1*) did not call attention to the possibility of cyclic fluctuations, their data suggested such an occurrence. More recently Maxon and Johnson (*10*) have confirmed the cycling phenomenon in a yeast propagator.

The present research concerns the propagation in the logarithmic phase of two aerobic bacteria, *Bacterium linens* and *Pseudomonas fluorescens;* cycling was also investigated, using a strain of *Saccharomyces carlsbergensis.*

Theory of Continuous Propagation With Logarithmic Growth

In batchwise cultivation single-celled organisms usually exhibit a phase of logarithmic growth which can be characterized by the equation

$$\frac{dN}{d\theta} = kN \qquad (1)$$

in which

N = the total number of cells
θ = time
k = a characteristic growth constant for the organism, which depends also on the environment—temperature, nature of medium, etc. Units are reciprocal time.

The rate constant, k, is conveniently measured by the slope of the growth curve when plotted on semilogarithmic coordinates.

$$k = \frac{(\text{slope})}{2.303} \qquad (2)$$

To achieve constant population in a flow system, the retention time within the propagator must equal $1/k$. Most investigators have been content simply to state this fact, but Monod (*12*) has provided rigorous proof based on a differential material balance.

By a material balance on the total cells, making use of Equation 1,

$$(\text{In} + \text{growth}) - \text{out} = \text{accumulation}$$

$$0 + kNd\theta - q\frac{N}{V}d\theta = dN \qquad (3)$$

in which V is the volume of liquid in the propagator and q is the volumetric flow rate.

[1] Present address, Corn Products Refining Co., Argo, Ill.

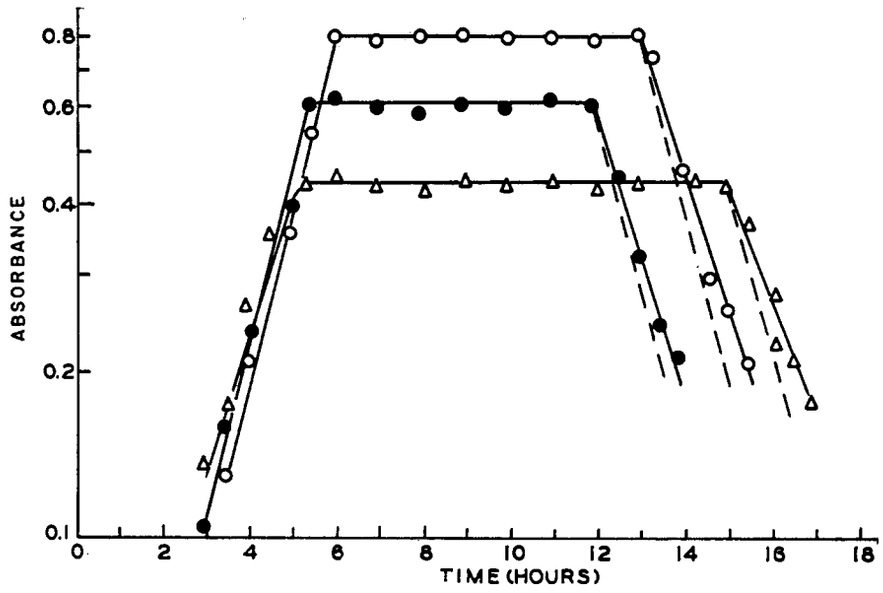


Figure 1. Verification of Equation 4 by substituting toxic reagents for nutrients being fed

$k = 0$
Three separate tests are shown. Substitution of phenol begun at 11.5 hours, of peroxide and sulfuric acid at 12.5 hours.
Ps. fluorescens. θ_r. 1.5 hours. Temperature, 23° C.
○ Saturated phenol
● 30% hydrogen peroxide
△ Concentrated sulfuric acid
--- Theoretical washout rates, Equation 4

From Equation 3 the rate of change of population is

$$\frac{dN}{d\theta} = N(k - q/V) \qquad (4)$$

An alternate form of Equation 4 is

$$\frac{dN}{d\theta} = N\left(\frac{\ln 2}{\theta_g} - \frac{1}{\theta_r}\right) \qquad (4a)$$

where θ_g is the familiar generation time of the organism, and θ_r is the mean retention time in the apparatus.

For the population to remain constant in a continuous propagator, the flow rate must be set so that

$$\theta_r = \frac{1}{k} = \frac{\theta_g}{\ln 2} \qquad (5)$$

If this condition is not met, the level of growth will change with time in accord with Equation 4.

It is assumed in the above analysis that flow rates are truly constant, and that mixing within the propagator is instantaneous and complete. The last assumption is generally not so severe a restriction as might be supposed (*9*).

Experimental

The propagator itself was not of unusual design. It consisted of a 500-cc. gas-scrubbing bottle appropriately modified to allow for inoculation and the subsequent feeding of nutrients. The overflow was of capillary glass tubing. The entire propagator was immersed in a water bath held at constant temperature, and there was provision for blowing sterile air at measured rates through a sintered-glass disk within the propagator. Dissolved oxygen, measured polarographically, was always in excess of the critical concentration for cellular uptake. No auxiliary agitation was required because of the high air rates used.

Of more interest to those wishing to study continuous fermentation was the use of a Sigmamotor pump (E and M Enterprises, Middleport, N. Y.) to provide constant flow rates of sterile nutrient. This pump has a cam arrangement, which moves fingerlike pieces of metal so as to press fluids through rubber or Tygon tubing, which passes between these fingers and a metal plate within the pump. Positive pumping at flow rates as low as 2 cc. per minute is easily possible. Changes in the flow rate may be made either by using rubber tubes of different size, or by modifying the speed of the pump. Freedom from contamination and ease of control recommend the use of such a pump over any variations of the Mariotte bottle as a means of attaining constant flow rates.

Growth was followed by measurements of absorbance (optical density), using a Coleman Nephocolorimeter (red filter No. 8-215). The linear response of the instrument was checked by plate counts, and when necessary dilutions were made before taking absorbance readings. The pH measurements were made with a Beckman line-operated meter. Sugar and carbon dioxide were measured by standard analytical methods.

The operating procedure was simple. To start the propagator, growth was allowed to proceed batchwise until, at an appropriate level of population,

Figure 2. Verification of Equation 4 by altering flow rate in two tests

B. linens. Temperature 26° C.
△ Curve 1
○ Curve 2

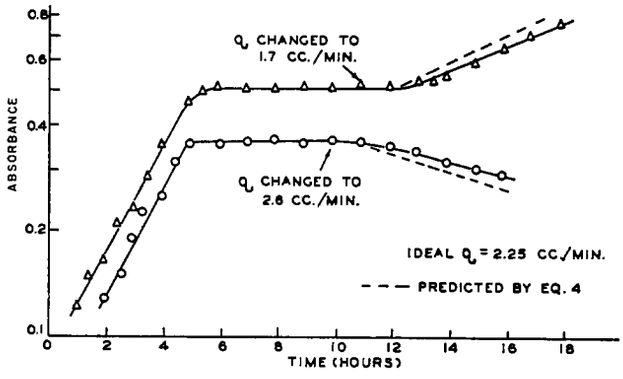
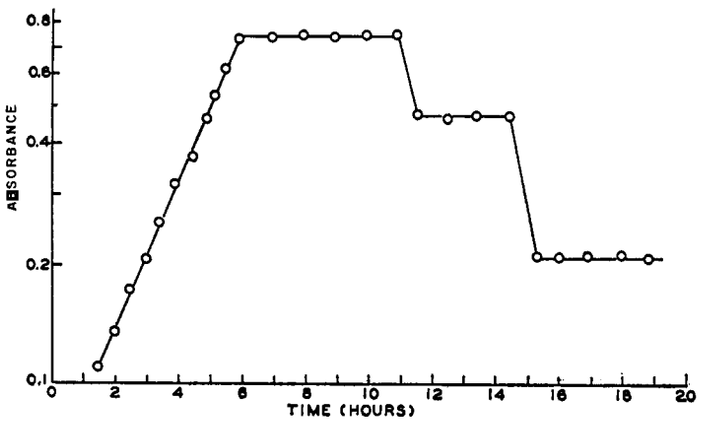


Figure 3. Verification of Equation 4 by altering population level

B. linens. Temperature 26° C. θ_r. 2.4 hours



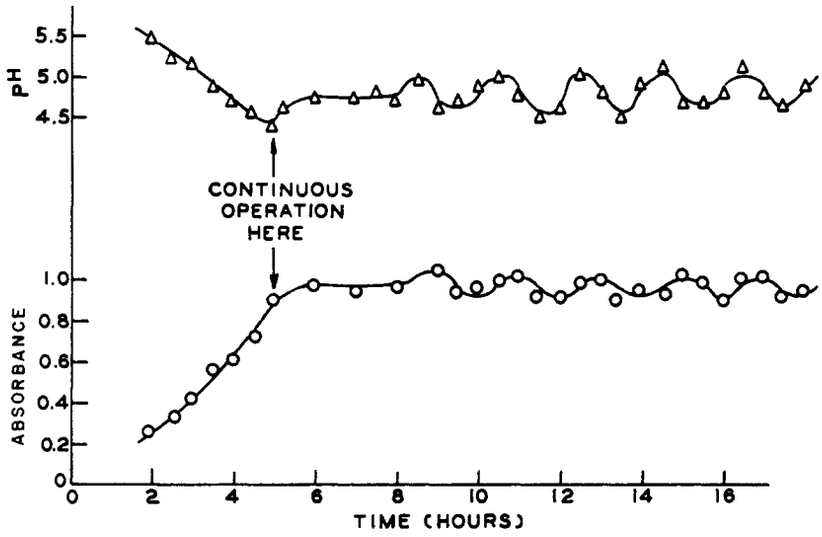


Figure 4. Example of steady cycling observed in continuous yeast propagator

S. carlsbergensis
Retention time 3.3 hours

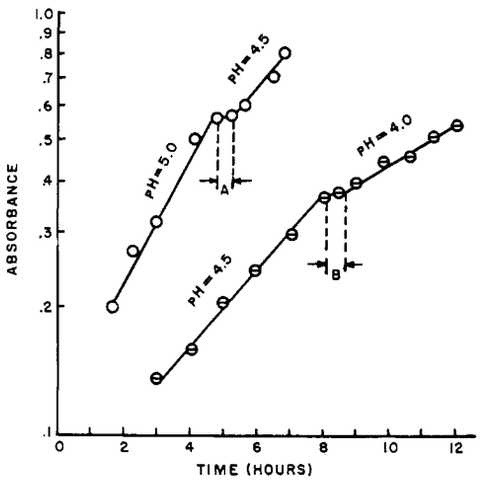


Figure 5. Effect of pH on growth rate

S. carlsbergensis. Retention time 3.3 hours
A. Lag 27 minutes
B. Lag 32 minutes
O Curve 1
θ Curve 2

continuous flow was begun. For *Ps. fluorescens* the medium was nutrient broth containing 1% glucose; for *B. linens* it was a tryptone-yeast extract broth with 0.5% glucose; for *S. carlsbergensis* the medium was nutrient broth with 2% glucose.

Performance of Propagator

Both *B. linens* and *Ps. fluorescens* exhibited logarithmic growth. Their behavior in the propagator is shown in Figures 1 to 3. The results are not so much a confirmation of the theory as a measure of the extent to which the particular setup satisfied the initial assumptions.

Of particular interest is Figure 3, which describes an experiment wherein normal operation was interrupted on two occasions by a rapid withdrawal of medium plus cells followed by replacement with fresh medium. Figure 3 demonstrates that for logarithmic growth, the population level is not uniquely determined by retention time in the propagator. Instead, the level of population depends only on the number of the cells present at the moment the continuous flow is started.

Experimental Studies on Cycling

The growth of yeast in glucose solutions does not proceed logarithmically for very long. One explanation for the short exponential phase is that sugar becomes converted into acid as well as into cell material; when the pH decreases, the rate of growth is slowed down also. Under such circumstances, a steady cycling of the population can be induced by altering the flow rate slightly from the value calculated from the slope of the growth curve.

All studies were made with *S. carlsbergensis*. During the period of cycling the pH, absorbance, glucose concentration, and carbon dioxide evolution were measured. However, only the absorbance and pH were followed in every run made. The results of typical experiments are shown in Figures 4 and 5.

The data are not sufficiently exact to establish the form of the oscillations, but for illustrative purposes simple sine waves have been drawn through the points, using a wave length of 2 hours. Curves for carbon dioxide evolution and sugar concentration are 180° out of phase with the curve for pH; this is in agreement with the findings of Maxon and Johnson (*10*).

A less obvious but more significant correlation from Figures 4 and 5 concerns the variation of pH with cell population. The two curves are not really in phase as reported by Maxon and Johnson (*10*), but instead, the pH curve lags the population curve by a quarter of a wave length. This fact gives a clue to the origin of the oscillations.

Cause of Steady Oscillations

In qualitative terms, one can account for the cycling as follows.

Imagine that the dynamic flow system is given some initial disturbance, such as a reduction in the flow rate, which causes the number of cells to increase in accord with Equation 4. As each cell manufactures acid the pH falls, and as the pH goes down, it causes a slowing up of the growth rate. It is not long before there is a net washout of cells, and the population decreases. Such a sequence of events will result in steady oscillation only if there is a time lag in the adjustments of pH and population.

In the interaction between the pH and the number of cells, it is idle to speculate on what is cause and what is effect, for the system contains a feedback mechanism similar to that encountered in electrical circuits. In fact, the system provides an interesting example of the "overshoot" phenomena which are so often encountered in biological systems, and which have been so well described by Burton (*5, 6*) and more recently by Denbigh *et al.* (*7*). Periodicities in chemical reactions and in biological reactions such as nerve excitation have been noted in the literature (*4, 13, 16*) but no comprehensive review is available and the references are widely scattered.

The classical studies of Volterra (*18*) and Lotka (*8*) on population dynamics apply the theory of relaxation oscillations to various cases of cycling. It is felt, however, that a clearer interpretation is made possible by applying the mathematical approach to automatic control problems presented by Minorsky (*11*). In this analogy, the yeast cells in the continuous propagator constitute a servo-system with inherent feedback and a finite time delay.

In order to describe the behavior of a yeast population, the basic differential equation of the propagator, Equation 4, must be modified to take into account the fact that k is not constant, but is a function of N. Owing to acid formation k decreases as N increases.

The following transformation is a convenient one:

$$N_L \equiv \ln \frac{N}{N_o} \qquad (6)$$

in which N_o is the steady population as set initially, and N represents a fluctuating value arising from a disturbance

applied to the system. Also, the right-hand member of Equation 4 may be abbreviated by

$$K \equiv k - \frac{q}{V} \qquad (7)$$

so that Equation 4 may be written

$$\dot{N}_L = K(N_L) \qquad (8)$$

This expresses the fact that k depends on N. Equation 8 can be expanded in a Taylor series, but for small values of N_L only the first two terms need to be retained, and

$$\dot{N}_L = K(N_L) = K_o - aN_L \qquad (9)$$

where a is a positive constant. Substitution of $N = N_o$ shows that K_o in Equation 9 is zero. Hence

$$\dot{N}_L + aN_L = 0 \qquad (10)$$

the solution of which is

$$N_L = N_L' e^{-a\theta} \qquad (11)$$

where N_L' is a small initial disturbance of the population. Equation 11 shows that the disturbance will always decay exponentially, and that there is no tendency for self-excited oscillations.

On the other hand, if there is a time delay in the adjustment, so that K depends upon the value of N_L at some previous time, stable oscillations are a natural consequence. Equation 10 may be written

$$\dot{N}_L + a\bar{N}_L = 0 \qquad (12)$$

where the bar above N_L signifies a retarded variable. If the time lag for adjustment is $\Delta\theta$,

$$\bar{N}_L = N_L(\theta - \Delta\theta) = N_L - \dot{N}_L\Delta\theta \ldots + \ddot{N}_L\frac{\Delta\theta^2}{2} + (-1)^n N_L^{(n)}\frac{\Delta\theta^n}{n!} \qquad (13)$$

Approximate solutions of Equations 12 and 13 are unreliable, but Minorsky (*11*) has provided an exact solution for such linear equations of infinite order:

$$N_L = N_L' e^{\alpha\theta} e^{i\omega} \qquad (14)$$

This equation describes a pulsation of frequency ω, and amplitude proportional to $e^{\alpha\theta}$. It suggests that it is the logarithm of the population which fluctuates sinusoidally. Baron (*2*), following Minorsky's treatment, has shown that for stable self-excited oscillations where the amplitude is independent of time

$$\omega = \frac{\pi}{2(\Delta\theta)}$$

or (15)

$$\Delta\theta = \frac{\lambda}{4}$$

where λ is the wave length in hours.

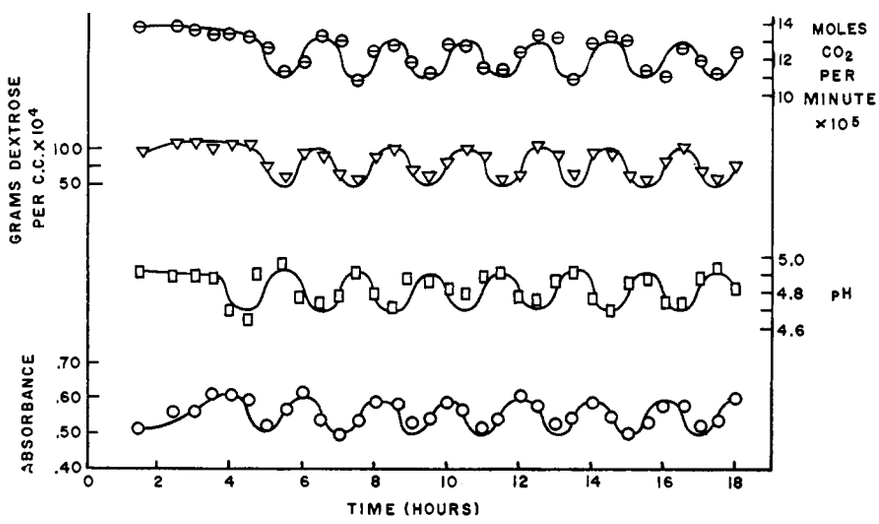
The time lag for *S. carlsbergensis* was, therefore, about 30 minutes (see Figures 4 and 5), which is much too long to be ascribed to any diffusional resistance. On the other hand, metabolic lags in adjustment to pH may be of this order, as shown by separate growth experiments with yeast.

In these experiments, the cells were grown batchwise at constant pH levels, which were controlled by the manual addition of acid or alkali; pH electrodes were immersed in the culture media, so that constant checks of pH could be made. After a logarithmic phase had become established, the pH level was changed abruptly by 0.5 unit, without appreciably diluting the medium. The results are shown in Figure 6. After a lag period of about 30 minutes, growth was resumed at a new rate. At pH 5.0 the generation time was 2.0 hours, compared to 2.5 hours at pH 4.5. Wilson (*20*) has shown that the magnitude of this difference in θ_g is of the right order to explain the extent of cycling in N. Nevertheless, other factors besides pH may also be influencing cell division.

These batch experiments failed to demonstrate any "overshoot," for the response to a change in pH was immediate. Growth did not continue unabated as if nothing had happened; instead, it stopped altogether for a time. Possibly the changes in pH were too sudden and too severe, but in any case the experiment showed that metabolic lags of the order of 30 minutes are involved.

Clearly much work remains to be done. The purpose of this investigation was simply to demonstrate that by critical analysis of data from a continuous propagator it is possible to find clues concerning metabolic behavior.

Figure 6. Growth of *S. carlsbergensis* in batch vessel at constant pH levels



Literature Cited

(1) Adams, S. L., and Hungate, R. E., *Ind. Eng. Chem.*, **42,** 1815–18 (1950).
(2) Baron, Thomas, "Proceedings of First Midwestern Conference on Fluid Dynamics," pp. 216–25, Ann Arbor, Mich., J. W. Edwards Co., 1951.
(3) Bilford, H. R., Scalf, R. E., Stark, W. H., and Kolachov, P. J., *Ind. Eng. Chem.*, **34,** 1406–10 (1942).
(4) Bonhoeffer, K. F., *J. Gen. Physiol.*, **32,** 69 (1948).
(5) Burton, A. C., *J. Cellular Comp. Physiol.*, **9,** 1–14 (1936).
(6) *Ibid.*, **14,** 327–49 (1939).
(7) Denbigh, K. G., Hicks, M., and Page, F. M., *Trans. Faraday Soc.*, **44,** 479–94 (1948).
(8) Lotka, A. J., *J. Am. Chem. Soc.*, **42,** 1595–9 (1920).
(9) MacDonald, R. W., and Piret, E. L., *Chem. Eng. Progr.*, **47,** 363–9 (1951).
(10) Maxon, W. D., and Johnson, M. J., Division of Agricultural and Food Chemistry, Bioengineering Symposium, 121st meeting, AM. CHEM. SOC., Milwaukee, Wis., 1952.
(11) Minorsky, N., *Appl. Mechanics*, **9,** A65–71 (1942).
(12) Monod, J., *Ann. inst. Pasteur*, **79,** 390–410 (1950).
(13) Moore, Margaret, *Trans. Faraday Soc.*, **45,** 1098–1109 (1949).
(14) Novick, A., and Szilard, L., *Proc. Natl. Acad. Sci. U.S.*, **36,** 708–19 (1950).
(15) Novick, A., and Szilard, L., *Science*, **112,** 715 (1950).
(16) Rashevsky, N., *Bull. Math. Biophys.*, **10,** 159–73 (1946).
(17) Unger, E. D., Stark, W. H., Scalf, R. E., and Kolachov, P. J., *Ind. Eng. Chem.*, **34,** 1402–5 (1942).
(18) Volterra, V., in Appendix of "Animal Ecology" by R. N. Chapman, New York, McGraw-Hill Book Co., 1931.
(19) Whittier, E. O., and Rogers, L. A., *Ind. Eng. Chem.*, **23,** 532–4 (1931).
(20) Wilson, R. E., Ph.D. thesis, University of Illinois (1953).

Received for review August 14, 1953. Accepted September 30, 1953. Presented before the Division of Agricultural and Food Chemistry at the 124th Meeting of the AMERICAN CHEMICAL SOCIETY, *Chicago, Ill. From a thesis submitted by R. E. Wilson to the graduate faculty of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy in chemical engineering.*