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Dense Autotrophic Cultures of *Alcaligenes eutrophus*

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Alcaligenes eutrophus was grown autotrophically in 23-liter batch cultures in a controlled H_2-O_2-CO_2 atmosphere. It was demonstrated that the need for periodic supplements of individual nutrients could be anticipated before cell growth depleted these nutrients to the point of becoming growth rate limiting. As a result, exponential growth was extended to optical densities of 44, with doubling times maintained at 2 h. Cultures having an initial optical density of 0.040 to 0.070 reached the final optical density of 60 in about 25 h. The final viable count was 1.2×10^{11} cells per ml, and the dry weight was 25 g/liter.

The unique ability of autotrophs to grow and reproduce in an exclusively inorganic medium generates a continuing interest in this group of microorganisms. Those autotrophs capable of rapidly converting CO_2 into cellular material have received special attention as potential sources of single-cell protein (13) and as bioregenerative life support systems for space travel (11, 12). One of the organisms studied was *Alcaligenes eutrophus*, formerly *Hydrogenomas eutropha* (7), which derives its energy for biosynthesis and growth from the oxidation of H_2. It does not require light and therefore can be grown in conventional large-volume fermentors. Required nutrients for *A. eutrophus* have been identified, and for most nutrients the minimum concentration required for exponential growth for a known increase in culture density has been determined (18).

This paper presents the successful application of these data in the production of high-cell-density batch cultures of 23-liter volume. Exceptionally high cell yields of 25 g (dry weight) per liter were obtained in a 25-h incubation period. The ease in obtaining these yields and the low cost of nutrients justify extending earlier studies that established the nutritional value of *A. eutrophus* protein for animal feeding (5, 20, 22). Synthesis of large amounts of poly-β-hydroxybutyrate (PHB) by this organism (19) and the ability to selectively initiate its production (18, 19) could be used advantageously to obtain high yields of this polymer.

MATERIALS AND METHODS

Stock cultures and inocula. *Alcaligenes eutrophus (Hydrogenomonas eutropha)* stock cultures and inocula were grown in 100-ml shaken cultures (15-17) in the modified medium described recently (18). Stock cultures could be stored for extended periods without losing viability (17). Inocula for the fermentor were grown in five or six 100-ml shaken cultures at 31°C and were harvested at the physiological age desired. The optical density (OD) of the pooled cultures was used to calculate the volume of inoculum needed for a starting OD of 0.040 to 0.070.

Fermentor. The 28-liter fermentor, a model MF-128S Microferm (New Brunswick Scientific Co., New Brunswick, N.J.) with a 23-liter working capacity, was designed for explosion-proof operation and was modified to accommodate a three-gas system. Commercial cylinder gases (H_2, O_2, and CO_2) were piped to the fermentor, where individual flow rates were established with calibrated flow meters (Brooks Instrument Division, Emerson Electric Co., Hatfield, Pa.). The gases were then sterilized by passing through steam-jacketed glass-wool filters. O_2 and CO_2 were combined in a common line (ball-check valves to prevent back flow) that entered the fermentor vessel through the head plate and terminated at a sparger under the impeller. A manually operated cutoff valve was located above the head plate. H_2 was delivered in a separate parallel system; mixing of H_2 with the other gases occurred in the fermentor vessel. A bypass outlet between the flow meters and the filters allowed gas flow rates to be established externally without distorting the gas atmosphere in the vessel. Percent composition of the gas atmosphere was based upon the ratio of the flow rate of a given gas to the total gas flow rate. During culture growth, the gases were continuously vented through the culture to an exhaust filter, a water trap, and finally into a ventillating exhaust duct. Distribution of gases in the fermentor vessel was facilitated by a variable-speed impeller with two paddle wheel blades.

The fermentor vessel and all gas lines downstream from the flow meters were stream sterilized in place; addition and sampling ports could be sterilized independently at any time during operation. Medium components, with the exception of phosphate buffer, were added aseptically; phosphate buffer (22 liters) was sterilized in the vessel at the time the fermentor was sterilized. Rapid cooling of

the buffer was accomplished by chilled water flowing through a closed loop in the vessel. An automatically determined chilled and hot water mixture flowing through the loop provided temperature control during incubation.

pH and O_2 electrodes were mounted through the head plate. The pH electrodes were connected to an automatic pH control unit (New Brunswick Scientific Co.) whose set point selector activated the appropriate peristaltic pump for acid or base addition. Interval timers regulated the pumping time and the delay time for mixing. Each operation (pulse) of the base addition pump activated a digital counter, which registered a cumulative pulse count, and an event marker (dissolved O_2 recorder), which indicated the frequency of addition. Dissolved O_2 was monitored with a sterilizable Clarke-type electrode (New Brunswick Scientific Co.) whose output was continuously recorded.

Fermentor medium. Twenty-two liters of 0.03 M potassium phosphate buffer, pH 6.5, was sterilized separately in the fermentor vessel (121°C for 1 h). After the incubation temperature of 31°C was reached, the other concentrated medium components in a total volume of 1 liter were added aseptically. These constituents and their final concentrations in the medium were: (i) NH_4Cl, 0.018 M; $CaCl_2$, 6×10^{-5} M; (ii) $NaHCO_3$, 0.012 M; K_2SO_4, 0.001 M; (iii) $MgCl_2$, 0.02 M; (iv) $FeCl_3$, 6×10^{-5} M; (v) $NiCl_2$, 5×10^{-7} M; (vi) $CuCl_2$, 4×10^{-7} M; (vii) $CrCl_3$, 2×10^{-7} M; (viii) $CoCl_2$, 2×10^{-7} M. The complete medium was equilibrated with the starting gas mixture for 30 min before inoculation. Necessary adjustments of the pH were made at this time. Individual nutrients were replenished during culture growth before they became growth rate limiting by the schedule discussed below. If an iron deficiency occurred, the resulting foaming was suppressed effectively with castor oil.

The total flow rate of the gaseous nutrients (H_2, O_2, and CO_2) was about 9 liters/min and was increased to 12 liters/min in denser cultures. Five percent O_2 in the starting mixture was maintained until the dissolved O_2 concentration decreased to 0.0048 mM (O_2 electrode). Subsequent increases in the O_2 flow rate or in the pO_2 were made as required to maintain the dissolved O_2 concentration between 0.0016 and 0.0048 mM. The CO_2 concentration in conjunction with the pH of the medium established the necessary bicarbonate concentration. The initial bicarbonate concentration was dictated by the physiological age of the inoculum; after 3 h of growth, the requirement was 6.6×10^{-4} M (17). This concentration was maintained in progressively denser cultures by correlating pCO_2 changes with pO_2 increases as discussed below.

Measurement of growth. Culture growth was monitored every 30 min by determining the optical density at 660 nm in a Gilford spectrophotometer with a standard cuvette (1-cm light path). Samples were diluted in 0.01 M phosphate buffer, pH 7, to give OD readings between 0.020 and 0.400. The relationship between OD, cell yields, and viable counts (48-h plate cultures on Trypticase soy agar or on autotrophic salts plus agar under H_2-O_2-CO_2) was consistent at low and at high cell densities. Each OD of 1 corresponded to 1.5×10^9 to 3×10^9 viable cells per ml. Cell yields were 1.3 to 1.55 g (wet weight)/liter per OD unit or 0.39 to 0.47 g (dry weight)/liter per OD unit, depending upon the PHB content of the cells. The dry weight averaged 30% of the Sharples centrifuged cell paste. Dry weight determinations were made by heating weighed portions of cell paste at 80°C until weights were constant for two consecutive 24-h periods.

RESULTS

Growth in 23-liter batch cultures with automatic pH control (NaOH) was the same as in 100-ml shaken cultures whose pH decreased to 5.5 or lower. Analysis of the spent medium showed both cultures were limited by ammonium-nitrogen, but a substantially higher NH_4Cl concentration could not be used because it inhibited growth (1, 15). The decrease in pH during growth was not caused by metabolic end products; it resulted primarily from progressive accumulation of unneutralized chloride ion as the ammonium ion was utilized. If the pH in the fermentor cultures was adjusted with NH_4OH (8 N) instead, the pH and the ammonium-nitrogen concentration simultaneously could be maintained. Such cultures, not restricted by NH_4^+ depletion, grew to OD 6 before another nutrient became limiting.

The successive depletion of various nutrients and the inability to anticipate these interruptions of growth reflected the absence of information about quantitative nutritional requirements of this organism. Qualitative requirements for macronutrients and some micronutrients were known (1, 3, 4, 6, 15–17), and some data were available on the relationship between total growth yield and the quantity of a nutrient. Virtually nothing was known about the critical concentration of individual nutrients at which exponential growth would become limited. An independent study was made (18) to determine the minimum saturating concentration of each nutrient needed for a given amount of culture growth. Multiples of these concentrations were tentatively assumed to support exponential growth to proportionally higher densities. Table 1 derived from these studies shows the relationship between the concentration of each required nutrient and the increment of exponential growth that can be obtained from that quantity of nutrient. The quantities shown can be added to the culture without adversely affecting growth. Although larger amounts of nutrients would extend the time before the next nutrient supplement was required, such excess amounts often produced inorganic precipitates or inhibition of growth.

TABLE 1. *Relationship between concentration of required nutrients and the increase in OD that can be expected before that nutrient becomes growth rate limiting*

Nutrient	Concn (mol/liter)	ΔOD[a]
PO_4^{3-}	3×10^{-2}	25
SO_4^{2-}	3×10^{-3}	24
NH_4^+	1.8×10^{-2}	3
Mg^{2+}	2×10^{-3}	16
Fe^{3+}	9×10^{-5}	7
Ni^{2+}	7.5×10^{-7}	8
Cu^{2+}	4×10^{-7}	9
Cr^{3+}	2×10^{-7}	8
Co^{2+}	2×10^{-7}	11

[a] Increment of exponential growth which can be obtained from the indicated concentration of nutrient.

The quantities recommended in Table 1 provide a margin of safety against premature depletion and are, therefore, more conservative than the experimentally derived values.

There is a discrepancy between the amount of Fe^{3+} and Ni^{2+} present in the medium at the time of inoculation and the amount added later in supplements. Both Fe^{3+} and Ni^{2+} at the higher concentrations interfered with initiation of growth; consequently, concentrations of 6×10^{-5} and 5×10^{-7} M, respectively, were used at the time of inoculation, and the remainder was added when the culture OD reached 1 to 2. Established cultures were inhibited by Ni^{2+} at 10^{-6} M.

Requirements for three additional trace metals, Cu^{2+}, Cr^{3+}, and Co^{2+}, were shown by their growth-stimulating ability (18), but quantitative requirements could not be determined because other medium components contributed unknown quantities of these metals. The nutrients listed in Table 1 plus any added indirectly in these reagents were sufficient to sustain rapid growth rates to high cell populations. A Zn^{2+} requirement had been reported for *A. eutrophus* (4), but we could not demonstrate a response to Zn^{2+} even after heavy culture growth. The significance of Zn^{2+} stimulation with 8-hydroxyquinoline-extracted medium in shaken cultures is not known (18).

As mentioned above, continuous ammonium ion supplements were automatically made by the pH control system. The net free ammonium ion concentration in the medium remained fairly constant at 0.009 M until high cell densities were reached. When the culture OD approached 40, the upper limit of 0.018 M free NH_4^+ was often exceeded, and it was necessary to substitute NaOH for NH_4OH. If at any time the lower limit of 0.003 M NH_4^+ excess was approached, a supplement of NH_4Cl was made.

The free NH_4^+ concentration in the medium was determined indirectly by the difference between the total milliequivalents of NH_4^+ added and the milliequivalents of NH_4^+ consumed to produce the observed cell density. The total NH_4^+ added to the medium was the sum of the NH_4^+ supplied as NH_4Cl plus the NH_4OH added by the pH control system. Cumulative NH_4OH addition was readily calculated since each operation (pulse) of the base addition pump delivered a known volume and concentration of NH_4OH, and the total number of pulses was registered on the digital counter. Ammonium ion consumed was estimated to be 138 meq for each optical density of 1.0 in 23 liters of medium. Culture growth could be monitored by inspection by the frequency of base addition shown by the pulse-activated event marker. The first indication of an impending nutritional deficiency was a decrease in the frequency of NH_4OH addition. If the deficiency was not corrected, NH_4OH addition stopped.

Early stages of a deficiency of certain nutrients could be recognized by characteristic signs. Either PO_4^{3-}, SO_4^{2-}, Mg^{2+}, or NH_4^+ limitation caused cell metabolism to shift to production of PHB (18). PHB vacuoles were visible microscopically as unstained areas in the cell cytoplasm. A deficiency of iron could be identified by culture foaming and at later stages by the appearance of a yellow-green pigment in the medium. Microscopically, iron-deficient cells stained uniformly but they appeared abnormally small.

Among the gaseous nutrients (H_2, O_2, and CO_2), H_2 required the least control; it was supplied in excess and was not limiting with as little as 50% H_2. Hydrogen bacteria are sometimes grouped according to O_2 sensitivity (14); some will not grow in 20% O_2. *A. eutrophus* grew well in shaken cultures with 10 to 25% O_2, but were O_2 limited at 5% O_2 (15). Five percent O_2 was only slightly limiting in 1-liter fermentor cultures, where gas distribution was more effective (17). Comparisons based upon pO_2 alone are, therefore, of doubtful validity because a variety of physical factors influence O_2 diffusion, which ultimately affect the rate of O_2 equilibration. These variables, even if held constant, eventually are perturbed by the increasing rate of O_2 demand in progressively denser cultures. The dissolved O_2 concentration at any moment must be determined by direct analysis (O_2 electrode). In the present studies the growth rate of *A. eutrophus* remained unchanged between 0.024 and 0.0016 mM O_2. During the first hours of growth the dissolved O_2 concentration was allowed to decrease from 0.024 to 0.0048 mM and thereafter was main-

tained between 0.0048 and 0.0016 mM, the equilibrium concentration with 3 to 1% O_2, respectively, at 31°C. These were unnecessarily low concentrations, but adjustments in carbon dioxide concentration had been empirically determined in reference to the lower range of O_2 concentrations as discussed below. The limiting dissolved O_2 concentration was not determined.

Growth of *A. eutrophus* specifically responded to the bicarbonate ion concentration, not to CO_2 per se, and a ±twofold difference in the optimum bicarbonate concentration caused the growth rate to decrease by more than 50% (17). In response to specific bicarbonate concentrations, "old" inocula will grow without a lag period at the same rate as exponential-phase inocula; the concentrations required were: 5.3×10^{-3} M for stationary-phase inocula, 1.6×10^{-3} M for postexponential-phase inocula, and 6.6×10^{-4} M for exponential-phase inocula. At pH 6.4, these concentrations were obtained with 16%, 5%, and 2% CO_2, respectively. After 1.5 cell doublings, or approximately 3 h, the old cells acquired the bicarbonate requirement of young cells, and the bicarbonate concentration had to be decreased to 6.6×10^{-4} M to maintain the initial rate of growth. Growth rates were not affected by pH changes between 6.4 and 7.2 if compensating changes in pCO_2 were made to keep a constant bicarbonate concentration. A CO_2 electrode was not available to monitor the CO_2 concentrations. After the bicarbonate concentration was established at 6.6×10^{-4} M, subsequent adjustments in pCO_2 were empirically determined by observing effects on the growth rate. Required increases in pCO_2 corresponded with increases in pO_2, and the ratio of the two gases remained constant at 1.8 to 2 (pO_2 to pCO_2) at a culture pH of 6.4. The growth rates in succeeding cultures were effectively maintained by basing pCO_2 changes on pO_2 changes.

Growth of a typical 23-liter batch culture is shown in Fig. 1. During the 25-h growth period, the OD increased 1,200-fold, from 0.050 to 60, corresponding to a final viable count of 1.2×10^{11} cells per ml. The cell yield was 25 g (dry weight)/liter (575 g/23 liters). Exponential growth with a doubling time of 2 h was maintained for the first 19 h to an OD of 44, at which time a break occurred, followed by a lesser exponential rate. The break in the growth rate occurred because of an NH_4^+ excess; NH_4OH automatically added in response to the pH drop exceeded the upper tolerance limit. A variety of technical difficulties usually became manifest when cultures reached high densities because of the large absolute increase in cells in a short time. For example, at OD 44, if the original

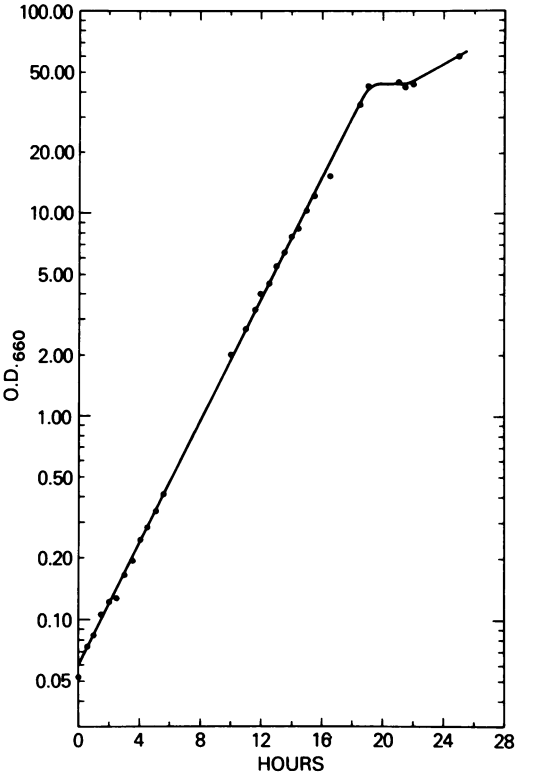

FIG. 1. *Autotrophic growth of A. eutrophus in 23-liter culture. Nutrient supplements and changes in H_2, O_2, and CO_2 flow rates were made as discussed in the text.*

growth rate continued for another 1 h, the anticipated OD would be 63. The 19-OD-unit increase required adjustments of gases and additions of nutrients that were too frequent for effective manual operation.

DISCUSSION

This work describes autotrophic growth conditions for *A. eutrophus* that have produced large-volume cultures of the highest density reported. A variety of heterotrophic bacteria have been grown to population densities of the same order of magnitude in culture volumes of 100 ml or less (9, 21). The restricted volumes were predicated in part by the complex media used and in part by the necessity to remove metabolic end products and replenish the medium by the dialysis technique. These limitations did not apply to *A. eutrophus* cultures because minimum nutritional requirements had been determined and the organism consumed nutrient without producing inhibitory metabolic by-products during autotrophic growth. As suggested by Tyrrell et al. (21), the

concept of biomass equilibrium-limiting microbial populations (2) is not justified. Microbial population limits are those which limit growth: availability of nutrients and removal or neutralization of inhibitory by-products.

Successful extension of *A. eutrophus* growth from culture densities of about OD 4 to OD 60 could not have been achieved unless qualitative and quantitative nutritional requirements (gases and salts) were known. The ability to maintain the exponential growth rate to optical densities of 44 indicated the quantitative requirements were satisfactorily anticipated, and the results validated the method of their determination (18). A 1.5- to 2-h doubling time represents the maximum capacity of the organism as determined by continuous and batch culture techniques (4). The nutritional data acquired with batch cultures should be directly applicable for determining nutrient feed rates in continuous cultures. It was noteworthy that in scaling up from 1- to 23-liter cultures, discrepancies in lag periods or in growth rates were not observed. Lag periods were eliminated by the same initial bicarbonate concentrations found effective in smaller volume cultures (17).

The final yield of 25 g (dry weight)/liter exceeded previously reported yields by a factor of 3 in a culture volume that here was eight times larger (4). These substantial yields do not represent maximum limits; it should be possible to sustain exponential growth to higher densities if mechanical manipulations such as feeding and gas flow rate adjustments are automated. Bongers (4) has already demonstrated the effectiveness of a closed gas system in which addition of individual gases was controlled by sensors. This type of system would greatly improve the efficiency of an open system in which gases are vented through the culture. The closed system has also been used with hydrogen and oxygen produced by electrolysis (8, 10).

An inexpensive, high-protein, single-cell food or food supplement is actively being sought to relieve the anticipated world demand for protein (13). Readily available inorganic nutrients are sufficient to produce high-protein *A. eutrophus* cells. The dry weight nitrogen content of *A. eutrophus* was 12 to 14%, of which 95% was accounted for as cell protein (5, 8, 18). The digestibility of this protein, as demonstrated in rat tests (5, 20), compared favorably with casein, and the biological value of the protein was the same as casein (5). Feeding tests with whole dead cells and whole cell sonic extracts showed *A. eutrophus* protein to be an effective food supplement for chimpanzees, dogs, swine, monkeys, rats, and mice (5, 22). Humans did not tolerate the *A. eutrophus* diet (22). For human consumption, it may be possible to develop a method to separate the protein from the endotoxic substance in the cell or, alternately, the whole material could be used directly as an inexpensive protein supplement in animal feed.

Another potential use of *A. eutrophus* cultures is in the production of PHB. PHB is preferentially synthesized from CO_2 when cultures become deficient in NH_4^+, PO_4^{3-}, SO_4^{2-}, or Mg^{2+} (18). Schlegel et al. (19) have employed a period of NH_4^+ starvation to induce synthesis of PHB and found as much as 65% of the cell mass to be PHB. This represents a considerable increase in PHB content, which normally is 1% or less in exponentially growing cells in a complete medium (18, 19). The total PHB yield could be increased significantly by withholding any of the four ions after high culture densities had been reached.

LITERATURE CITED

1. **Atkinson, D. E.** 1955. The biochemistry of *Hydrogenomonas*. III. The effect of inorganic nitrogen compounds on hydrogen uptake. J. Bacteriol. 70:78–81.
2. **Bail, O.** 1929. Ergebnisse experimenteller Populationsforschung. Z. Immunitaetsforsch. 60:1–22.
3. **Bartha, R., and E. J. Ordal.** 1965. Nickel-dependent chemolithotrophic growth of two *Hydrogenomonas* strains. J. Bacteriol. 89:1015–1019.
4. **Bongers, L.** 1970. Some aspects of continuous culture of hydrogen bacteria, p. 241–255. *In* C. J. Corum (ed.), Developments in industrial microbiology, vol. 2. Garemond-Pridemark Press, Baltimore.
5. **Calloway, D. H., and A. M. Kumar.** 1969. Protein quality of the bacterium *Hydrogenomonas eutropha*. Appl. Microbiol. 17:176–178.
6. **Cohen, J. S., and R. H. Burris.** 1955. A method for the culture of hydrogen bacteria. J. Bacteriol. 69:316–319.
7. **Davis, D. H., M. Doudoroff, R. Y. Stanier, and M. Mandel.** 1969. Proposal to reject the genus *Hydrogenomonas*: taxonomic implications. Int. J. Syst. Bacteriol. 19:375–390.
8. **Foster, J. F., and J. H. Litchfield.** 1964. A continuous culture apparatus for the microbial utilization of hydrogen produced by electrolysis of water in closed-cycle space systems. Biotechnol. Bioeng. 6:441–456.
9. **Gerhardt, P., and D. M. Gallup.** 1963. Dialysis flask for concentrated culture of microorganisms. J. Bacteriol. 86:919–929.
10. **Gottschalk, G., and H. G. Schlegel.** 1965. Growth of 'knallgas' bacteria (*Hydrogenomonas*) using direct electrolysis of the culture medium. Nature (London) 205:308–309.
11. **Jenkins, D. W.** 1966. Bioregenerative life-support systems, p. 1–6. *In* Bioregenerative systems, NASA SP-165. U.S. Government Printing Office, Washington, D.C.
12. **Jenkins, D. W.** 1966. Electrolysis-*Hydrogenomonas* bacterial bioregenerative life support system, p. 229–244. *In* Proceedings of the XVIth International Astronautical Congress, Athens, Greece. Gordon and Breach, New York.
13. **Mateles, R. I., and S. R. Tannenbaum (ed.).** 1968. Single cell protein. The M.I.T. Press, Cambridge, Mass.
14. **Packer, L., and W. Vishniac.** 1955. Chemosynthetic

fixation of carbon dioxide and characteristics of hydrogenase in resting cell suspensions of Hydrogenomonas ruhlandii nov. spec. J. Bacteriol. 70:216-223.
15. **Repaske, R.** 1962. Nutritional requirements for *Hydrogenomonas eutropha*. J. Bacteriol. 83:418-422.
16. **Repaske, R.** 1966. Characteristics of hydrogen bacteria. Biotechnol. Bioeng. 8:217-235.
17. **Repaske, R., C. A. Ambrose, A. C. Repaske, and M. L. DeLacy.** 1971. Bicarbonate requirement for elimination of the lag period of *Hydrogenomonas eutropha*. J. Bacteriol. 107:712-717.
18. **Repaske, R., and A. C. Repaske.** 1976. Quantitative requirements for exponential growth of *Alcaligenes eutrophus*. Appl. Environ. Microbiol. 32:585-591.
19. **Schlegel, H. G., G. Gottschalk, and R. V. Bartha.** 1961. Formation and utilization of poly-β-hydroxybutyrate by knallgas bacteria (*Hydrogenomonas*). Nature (London) 191:463-465.
20. **Shapira, J., and A. D. Mandel.** 1968. Nutritional evaluation of bacterial diets in growing rats. Nature (London) 217:1061-1062.
21. **Tyrrell, E. A., R. E. MacDonald, and P. Gerhardt.** 1958. Biphasic system for growing bacteria in concentrated culture. J. Bacteriol. 75:1-4.
22. **Waslien, C. I., D. H. Calloway, and S. Margen.** 1969. Human intolerance to bacteria as food. Nature (London) 221:84-85.