

Effect of Bacterial Density and Substrate Concentration on Yield Coefficients

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Measurements were made of the yield coefficient during the aerobic metabolism of glucose by a heterogeneous bacterial mixture. Expressed in terms of carbon, the coefficient was approximately 0.48. The value did not vary with initial bacterial densities ranging from 0.4 pg to 40 µg of cell carbon per ml and with glucose concentrations ranging from 43 pg to 100 µg of carbon per ml. Under all these circumstances, about 44% of the glucose carbon was converted to CO₂, and 7.4% was excreted as organic products. The significance of uncharacterized organic substrates contaminating the medium to the coefficients calculated for low glucose concentrations is discussed.

Studies of the efficiency of biomass production at the expense of energy and carbon source, i.e., the yield coefficient, are important for the analysis of both the kinetics of microbial processes in culture (9, 13) and the conversion of substrates in natural ecosystems to biomass, organic products, and CO₂ (6, 8). Large differences exist among values reported for the yield coefficient, however, and these variations make difficult the analysis of processes in culture and of the conversions in natural environments.

Differences in values for the coefficient have been explained as resulting from the dissipation of cell carbon by maintenance metabolism (9), dissimilar metabolic pathways (1, 10), the energy content of the substrate (7), the level of dissolved oxygen (5), the pH of the culture (12), and the temperature (11). Most of these explanations are based on the results of experiments in which microbial densities, substrate concentrations, or both are far higher than those generally found in natural environments.

The present study was designed to determine the yield coefficients of a heterogeneous bacterial mixture, with special reference to the variables of cell density and substrate concentration. Particular attention was given to determining the yield coefficients at trace concentrations of the organic substrate because low levels of numerous synthetic compounds, for example, less than 100 ng/ml, characterize the concentration of pollutants in many fresh, estuarine, and marine waters.

MATERIALS AND METHODS

Sewage from the primary settling tank of the sewage treatment plant of Ithaca, N.Y., was passed through a 3.0-µm-pore membrane filter (Millipore Corp., Bedford, Mass.), and 0.5-ml portions were added to 500 ml of an inorganic salts solution. The cell suspension thus prepared was maintained by adding 0.5 mg of tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) to the solution every 5 to 7 days. After 1 to 2 months, the cell suspension was again filtered and diluted 100-fold, and 0.7-ml portions were added to 70 ml of sterilized inorganic salts solution contained in 200-ml Erlenmeyer flasks supplemented with one of five different amounts of TSB to provide five different initial cell

densities. The two filtrations were designed to remove protozoa, which were not found by microscopic examination of the final mixtures of bacteria. The flasks were stoppered with silicone foam plugs and incubated at 24°C on a reciprocal shaker, except that flasks containing both low concentrations of substrate and low densities of bacteria were sometimes incubated without shaking.

The inorganic salts solution contained: 0.9 mM KH₂PO₄, 2.1 mM Na₂HPO₄, 2.0 mM NH₄NO₃, 0.1 mM MgSO₄ · 7H₂O, 0.1 mM CaCl₂ · 2H₂O, 2 µM FeCl₃ · 6H₂O, 10 µM MnCl₂ · 4H₂O, 10 µM H₃BO₃, 10 µM Na₂MoO₄ · 2H₂O, 0.05 µM CuSO₄ · 5H₂O, 0.05 µM ZnSO₄ · 7H₂O, 0.05 µM CoSO₄ · 7H₂O, 0.1 mM Na₂SiO₃ · 9H₂O, and 50 mM NaCl. The pH was 7.3. The solutions were prepared with distilled water that had been passed through the Milli-Q reagent-grade water system (Millipore Corp.). The glassware was soaked for 30 min at 70°C in 1.0% (wt/vol) K₂S₂O₈ solution and then rinsed three or four times with the reagent-grade distilled water.

For counting bacteria, the pour-plate method was used after diluting the samples in the inorganic salts solution. The counting medium contained 0.1% TSB powder, 1.2% agar, and the salts solution. For the estimation of bacterial dry weight, the suspension was passed through 0.2-µm-pore filters (VWR Scientific, Inc., Rochester, N.Y.), and then the filters were dried and weighed. Based on the counts and measurements of dry weight, values of 0.2 pg of dry weight and 0.1 pg of carbon per cell were used when the cell density was low.

In studies in which glucose was added to the cultures, a medium containing 100 pg of D-[U-¹⁴C]glucose (specific activity, 333 mCi/mmol; New England Nuclear Corp., Boston, Mass.) per ml and unlabeled glucose to the desired final concentration was used. At intervals, 5.0 ml of suspension was removed from the culture, and a 2.0-ml portion was placed in a scintillation vial. The remainder was passed through a 0.22-µm-pore membrane filter (type GS; Millipore Corp.), and 2.0 ml of the filtrate was placed in another vial. Concentrated H₃PO₄ (2 drops) was added to each of the vials, which then were allowed to stand for 24 h, with occasional shaking to expel ¹⁴CO₂. Then, 12 ml of ACS scintillation cocktail (Amersham Corp., Arlington Heights, Ill.) was added to each of the vials. The radioactivity was counted for 10 min with a liquid scintillation counter (model LS 7500; Beckman Instruments, Inc., Irvine, Calif.).

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The amount of newly formed biomass was calculated from the difference in the radioactivities before and after the suspension was filtered. The yield coefficient was expressed as the ratio of carbon in the newly formed biomass to the carbon in the consumed glucose; the values were determined in the early stationary phase, when it was assumed that the glucose had been entirely metabolized.

RESULTS

The mixture of bacteria was grown in the inorganic salts solution containing various concentrations of TSB powder. The rates of growth were slowest in media containing 0 and 0.02 μg of TSB powder per ml and were greater at the three higher levels (Fig. 1). The CFUs (10^6) in the stationary phase were 0.48, 0.43, 4.3, 45, and 380 per ml at 0, 0.02, 2.0, 20, and 200 μg of TSB per ml. Thus, the numbers were proportional to the concentrations of TSB except for the lack of difference between the unamended solution and the medium with 0.02 μg of TSB per ml. Based on this proportionality, the uncharacterized organic compounds in the salts solution were estimated to be equivalent to 0.2 μg of TSB per ml. A similar experiment with glucose added to the salts solution indicated that uncharacterized organic compounds in the solution were nutritionally equivalent to ca. 0.1 μg of glucose carbon per ml. The dry weight of cells in the stationary phase in the culture grown on 200 μg of TSB per ml was 79 $\mu\text{g}/\text{ml}$; if it is assumed that the cells have 50% carbon, this dry weight represents ca. 40 μg of carbon per ml. Cultures in the stationary phase contained four or five different colony types.

Labeled and unlabeled glucose with a final concentration of 1.0 μg of carbon per ml were added to 72-h cultures equivalent to those shown in Fig. 1, and the radioactivity in the cells and the solution was measured at regular intervals.

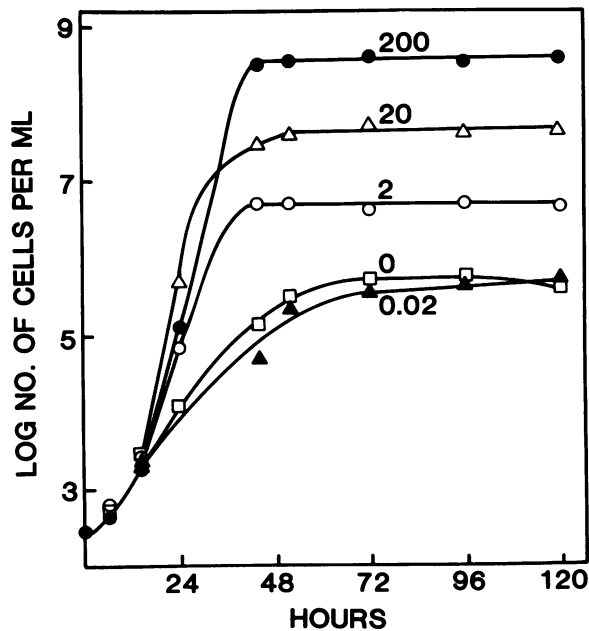


FIG. 1. Growth of the heterogeneous bacterial mixture in unamended salts solution and in salts solution with four concentrations of TSB powder. The values are the initial TSB levels, measured in micrograms per milliliter.

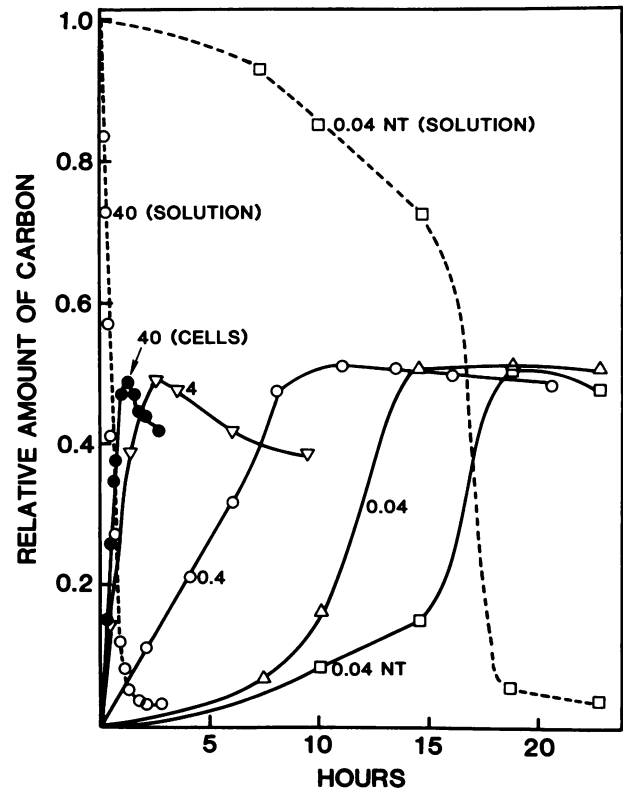


FIG. 2. Metabolism of glucose (1.0 μg of carbon per ml) at five bacterial densities (given as micrograms of carbon per milliliter). NT represents cells grown in a TSB-free medium, in which no carbon was deliberately added. The y axis gives the ratio of the radioactivity in the newly formed biomass or in the solution to the initial radioactivity.

The amount of ^{14}C incorporated into the cells increased with time, and this was accompanied by a decrease in the radioactivity of the solution (Fig. 2). Only two of the curves are presented for the ^{14}C levels in the solution. As expected, the higher the cell density, the less time was required to reach maximum ^{14}C incorporation. It is noteworthy that although two treatments had the same initial cell density (equivalent to 0.04 μg of carbon per ml), the cells grown only on contaminating carbon sources in the salts solution took longer to incorporate ^{14}C than did the cells grown in the presence of 0.02 μg of TSB per ml, a medium that contains glucose.

The yield coefficients were determined in the early stationary phase, during which time the maximum incorporation was observed, the assumption being that the low radioactivities in the solutions at this time represented metabolic products rather than residual glucose. The yield coefficients were almost identical, and the mean value of the five determinations was 0.50, even though the initial bacterial densities differed by a factor of 1,000. It should be noted that the ^{14}C was incorporated, but no tests of net increases in biomass in the presence of glucose were made.

A study was conducted to determine the yield coefficients at four lower bacterial densities. The glucose level was 1.0 μg of carbon per ml. The initial population densities were equivalent to 400, 40, 4, and 0.4 μg of carbon per ml, and the times to reach maximum incorporation were 32, 50, 62, and 74 h, respectively. Under these conditions, the mean value for the yield coefficients was 0.49.

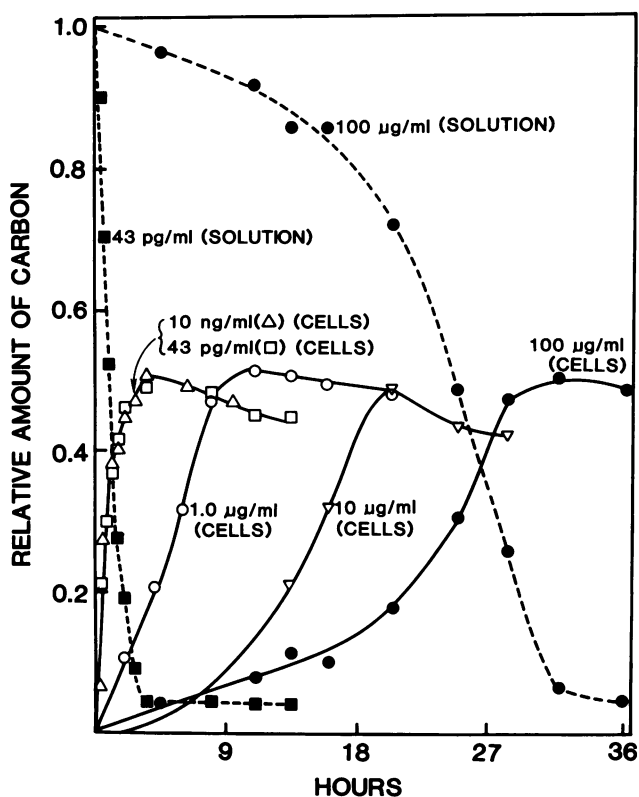


FIG. 3. Metabolism of 43 pg, 10 ng, 1.0 μg , 10 μg , and 100 μg of glucose carbon per ml by bacteria at a density of 0.4 μg of carbon per ml. The y axis is the same as that in Fig. 2.

A 72-h culture grown in a medium with 2.0 μg of TSB per ml was amended with glucose to nine different concentrations ranging from 43 pg to 100 μg of carbon per ml. The bacterial biomass was estimated to be 0.4 μg of carbon per ml. The time to reach the maximum ^{14}C incorporation increased as the glucose concentration rose, but the actual rates of incorporation probably were higher at the higher sugar levels (Fig. 3). A total of nine glucose concentrations were tested, but only two curves for the ^{14}C remaining in the solution and five curves for ^{14}C incorporation are shown. Although the glucose concentrations differed by more than 10^6 -fold, the yield coefficients were almost identical. The mean value of the nine determinations was 0.49.

The yield coefficients were also determined by using a cell biomass of 40 pg of carbon per ml and glucose concentrations of 1.0 and 100 μg of carbon per ml. The times to attain maximum incorporation were 49 and 66 h, and the yield coefficients were 0.48 and 0.49, respectively. It is likely that growth occurred during the test period, so the yield coefficients represent growing cells.

To study the effect of starvation, a 240-h culture was used, but the other experimental conditions were similar to those in the experiment described in Fig. 3. The bacterial biomass was estimated from the number of CFUs to be 0.4 μg of carbon per ml. Nine glucose concentrations were tested, but only representative data are presented. The incorporation of ^{14}C and its loss from solution are shown in Fig. 4. The mean value of the nine yield coefficients was 0.47.

To investigate the metabolism of extremely low substrate concentrations by large cell numbers, a study was conducted

of the biodegradation of 43 pg of glucose carbon per ml by cells at a density of 40 μg of carbon per ml. The ^{14}C disappeared rapidly from the solution and was incorporated into the biomass (Fig. 5), which had ca. 10^6 times more carbon than the substrate. This initially rapid loss of ^{14}C from the solution may be a result of absorption by the biomass or its accumulation in intracellular pools, rather than the biodegradation of the glucose. After the initially rapid uptake by the bacteria, the radioactivity of the biomass declined to a value with a yield coefficient of 0.48 at 1.75 h. At 9 h, 34% of the added radioactivity was still retained in the biomass.

When glucose at 100 ng of carbon per ml was supplied to bacteria at a density of 4.0 or 40 μg of carbon per ml, this rapid absorption was not observed (data not shown). The yield coefficients were 0.54 at 0.18 h for the higher cell density and 0.55 at 0.7 h for the lower cell density.

From the 40 measurements made in this study, the mean, standard deviation, and the coefficient of variation of the yield coefficient were calculated. The mean values for carbon converted to biomass, CO_2 , and organic products were also determined. All values were determined for cultures in the early stationary phase of growth. Assuming that all of the glucose had disappeared at this time, the loss of ^{14}C from the culture (after the addition of H_3PO_4) was considered to reflect CO_2 formation, and the radioactivity remaining in the filtrate was considered to reflect the yield of organic products. The values are presented in Table 1. The small variation in the yield coefficients and the percentage of carbon converted to CO_2 suggest that bacterial density, substrate concentration, and starvation do not affect the yield coefficient or the extent of mineralization.

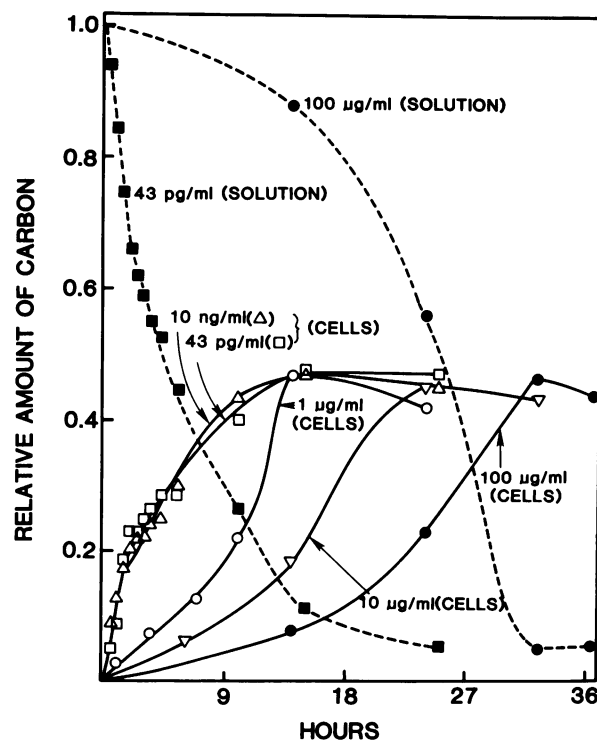


FIG. 4. Metabolism of 43 pg, 10 ng, 1.0 μg , 10 μg , and 100 μg of glucose carbon per ml by starved bacteria at a density of 0.4 μg of carbon per ml. The y axis is the same as that in Fig. 2.

DISCUSSION

A major purpose of this study was to determine the yield coefficients at low concentrations of organic substrates. At low substrate concentrations, it might be expected that the values would be low because of the slow growth rates and the use of cell carbon to satisfy the needs for maintenance metabolism (9, 14). In the present investigation, however, the yield coefficient at the expense of glucose was almost the same as those obtained in studies in which the substrate concentrations were relatively high (7, 11, 12, 16). It is noteworthy that Chesney et al. (2) reported that 20 to 25% of the carbon from phenol added to pond water at concentrations of 1 to 1,000 ng/ml was incorporated into the biomass.

It has been reported that starvation and a change in growth rate from low to high in cultures grown in chemostats lowered the yield coefficients (4, 14). In the present study, however, starvation had no effect on the coefficient. In a batch culture in which the bacterial population is low and the added substrate concentration is high, it is likely that the effect of starvation is overcome in fresh media because of the added glucose. At very low concentrations of glucose, the amount of glucose may be too small to alleviate the effects of starvation, but the level of uncharacterized organic contaminants in solution may be enough to do so. Therefore, the coefficients observed at these low concentrations do not really reflect the effects of starvation and are the same as those observed at high concentrations of glucose; that is, the

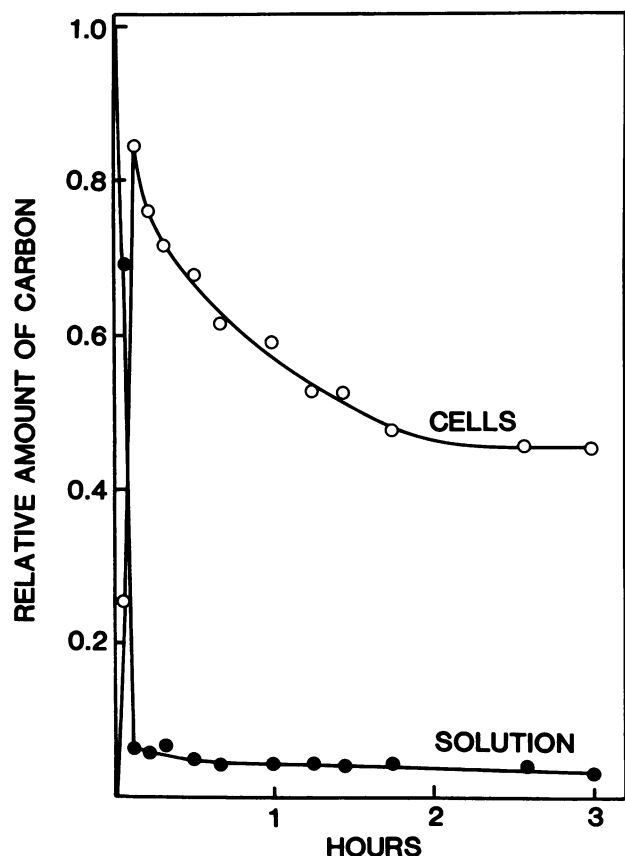


FIG. 5. Metabolism of 43 pg of glucose carbon per ml by bacteria at a density of 40 μg of carbon per ml. The y axis is the same as that in Fig. 2.

TABLE 1. Yield coefficient and carbon balance in cultures containing a mixture of bacteria incubated with glucose

Value measured	Mean	SD	Coefficient of variation
Yield coefficient	0.48	0.03	6.3
Carbon balance (%)			
Biomass	48.2	3.08	6.3
CO ₂	44.3	3.78	8.5
Organic products	7.4	2.72	36.5

observed coefficients may not reflect the effects of low glucose concentrations but rather the combined influence of glucose and uncharacterized organic contaminants.

The rate of entry of airborne organic carbon through silicone rubber stoppers into a sterile solution has been reported to be 1.2 ng/ml per h (3). In contrast, the maintenance coefficients range, for example, from 0.04 to 0.001 per h (6). If the airborne organic carbon entering a medium at a rate of 1.2 ng/ml per h was completely used by a bacterial population with a biomass of 0.4 μg of carbon per ml, it follows that the maintenance coefficient (9) was 0.003/h, which is in the range cited above. Therefore, further studies with highly purified water or with organisms unable to use the contaminating carbon will be necessary to obtain reliable yield estimates at very low substrate concentrations.

It has been reported that the yield coefficient for an estuarine community was 0.64 at glucose concentrations lower than 10^{-7} M and 0.51 at concentrations of 10^{-5} and 10^{-4} M. This suggests the simultaneous use of naturally occurring substrates in the water when the concentration of the added substrate is low, and the use and extensive metabolism of the single substrate when its concentration is high (16). If the nutritional values of the ambient substrates were the same with added glucose, the coefficient would not vary with change in glucose concentration.

Observations that 80 to more than 90% of the carbon from substrates added at low concentrations to fresh waters are converted to CO₂ (15) differ from the results of this study. The almost complete mineralization may result from the inability of the organism active on the test chemical to use much of the other organic compounds in the water, so that nearly all of the carbon in the test compound is used to satisfy the need of the organisms for maintenance and thus is converted to CO₂. On the other hand, the constancy in yield at low substrate concentrations, as reported here, may result from the ability of the glucose-metabolizing species to use much of the other forms of organic carbon in the water for endogenous respiration, so that declines in yield coefficients arising from maintenance demands are not evident.

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