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Kinetics of Two Subgroups of Propionate-Using Organisms in Anaerobic Digestion

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A novel method for measuring the kinetics of a subgroup of organisms growing in mixed culture was used to measure the kinetics of propionate-using organisms in a mixed-population anaerobic digester. It was shown that there are at least two subgroups of propionate-using organisms with distinctly different growth kinetics. Both subgroups could grow without reducing sulfate. The slower-growing subgroup had a K_s of 11 mg/liter and a μ_{max} of 0.0054 h^{-1} which is similar to the μ_{max} reported for *Syntrophobacter wolinii*. The faster-growing group had a μ_{max} of 0.050 h^{-1} and a K_s of 330 mg/liter. The slower-growing group was inhibited by a pH shock from 7.0 to 6.0, whereas the faster-growing group was less sensitive to the pH shock.

Propionate-using organisms appear to play an important role in the sequence of events leading to digester failure under overload conditions. It has been reported that digester organisms are sensitive to high concentrations of propionate (1, 5) and that propionate-using organisms are sensitive to hydrogen concentration (7; P. S. Shuba, Ph.D. thesis, University of Florida, Gainesville, 1973), acetate concentration (7), and carbon dioxide concentration (4). To include the role of propionate-using organisms in a model of an anaerobic digester, further information on the growth kinetics and pH sensitivity of these organisms is required.

Most anaerobic digesters contain insufficient sulfate in the feed for sulfate-reducing propionate users such as *Desulfobulbus propionicus* (10) to form a major part of the propionate-using subgroup. A propionate-using organism that does not need to reduce sulfate itself has been isolated (3) and grown in coculture with hydrogen-using sulfate reducers and methanogens, but it is still too early to establish whether *Syntrophobacter wolinii* is the major propionate-degrading bacterium in anaerobic digesters, or even typical of propionate-degrading bacteria in its growth kinetics. Consequently, it is important to compare the growth kinetics of *S. wolinii* with the growth kinetics of propionate-degrading organisms that occur naturally in mixed-population digesters.

For *S. wolinii* grown in coculture with a sulfate reducer at 35°C, a minimum doubling time of 87 ± 7 h (maximum specific growth rate [μ_{max}] = 0.008 h^{-1}) was reported, whereas a minimum doubling time of 161 ± 18 h (μ_{max} = 0.004 h^{-1}) was measured for growth in coculture with a methanogen (3). From a continuous enrichment-culture digester allowed to reach a series of steady states, Lawrence and McCarty (9) obtained an equivalent μ_{max} value of 0.017 h^{-1} with a K_s value of 32 mg/liter. The digesters were kept at 35°C and fed a mineral salts medium including 6×10^{-4} mol of sulfate (A. W. Lawrence, Ph.D. thesis, Stanford University, Stanford, Calif., 1967). This is less than 1% of the amount of sulfate needed to react with the total hydrogen produced from the feeds (1,560 and 3,010 mg of propionate per liter), so that the growth rate should be compared with the growth of *S. wolinii* in coculture with a methanogen.

There is a considerable difference between the maximum specific growth rate of *S. wolinii* without sulfate (0.004 h^{-1}) and the maximum specific growth rate for the mixed-culture propionate users (0.017 h^{-1}). This suggests that the group of organisms using propionate is not homogeneous. However, the method used by Lawrence and McCarty (9) is based on the assumption that the propionate-using organisms can be treated as a homogeneous group. As such, the kinetics obtained by Lawrence and McCarty include the effects of population changes within the propionate-using subgroup and should be recognized as "balanced" kinetics. Since changes in population in a digester would be expected to occur slowly, these kinetics cannot be expected to describe a transient response. Furthermore, balanced kinetics could not be expected to reflect the kinetics of any one digester organism. These distinctions apply only if population changes within the subgroup result in

significant variation in the kinetics of the subgroup.

The "transient" kinetics of a subgroup is defined as the response of the subgroup to changes in medium conditions in a time that does not allow significant variation in population to occur. If a particular organism predominates in a subgroup under certain conditions, then the transient kinetics of the subgroup measured in a digester acclimatized to those conditions should be similar to the kinetics of the predominant organism. If the transient kinetics of the subgroup are essentially the same when measured in digesters at different retention times, then the subgroup can be described as kinetically homogeneous, and the balanced and transient kinetics should be the same. To measure the kinetics of propionate users without assuming that propionate users could be treated as a homogeneous group over a wide range of digester retention time selection pressures, we had to develop a new method of establishing kinetics within mixed cultures.

Kinetic analysis in mixed culture. The difficulty of obtaining μ_{max} and K_s values for subgroups within mixed-population digesters is largely a problem of estimating the cell mass of the particular subgroup being studied. Some effort has been made to estimate the fraction of cell mass of a subgroup by using thermodynamic considerations (9), whereas other workers have avoided the problem by measuring v_{max} in terms of moles of propionate used per liter per hour (8). Measurement of v_{max} is of little use for modeling since v_{max} will vary according to the mass of the subgroup present in the mixed culture. The following analysis technique allows measurement of K_s and μ_{max} for a subgroup in mixed culture without the necessity of estimating the fraction of cell mass represented by the subgroup. It is assumed that the organism decay rate is negligible (see below).

The mass balance equation for any substrate supplied in the feed to a continuous stirred tank reactor is as follows: rate of accumulation of substance in fermenter = rate of input in feed stream − rate of output in outlet stream − rate of substrate uptake, or

$$\frac{dS}{dt} = v\,C_{so} - v\,C_{sf} - Q_s X \qquad (1)$$

where S is the mass or moles of substrate in the fermenter, v is the volumetric feed rate to the fermenter, C_{so} is the concentration of substrate in the feed, C_{sf} is the concentration of substrate in the product, Q_s is the substrate uptake rate per unit of biomass, and X is the cell mass in the fermenter.

Dividing throughout by the fermenter volume, the equation is expressed in its usual form:

$$\frac{dC_s}{dt} = D(C_{so} - C_{sf}) - Q_s C_x \qquad (2)$$

where D is the dilution rate and C_x is the concentration of biomass in the fermenter.

If μ is defined as the rate of increase in biomass per unit of biomass, and it is assumed that the growth yield is a constant equal to Y (gram of cells per gram or mole of substrate), then it follows that:

$$Q_s = \frac{1}{Y}\mu \qquad (3)$$

Substituting this into equation 2 gives:

$$\frac{dC_s}{dt} = D(C_{so} - C_{sf}) - \frac{1}{Y} \cdot \mu \cdot C_x \qquad (4)$$

Similarly, mass balance on biomass gives (assuming no biomass in the feed):

$$\frac{dC_x}{dt} = C_x(\mu - D) \qquad (5)$$

Substituting equation 5 into equation 4 gives:

$$\frac{dC_s}{dt} = D(C_{so} - C_{sf}) - \frac{1}{Y}\left(\frac{dC_x}{dt} + DC_x\right) \qquad (6)$$

At steady state, $dC_x/dt = 0$, so from equation 5 we have the usual result:

$$D = \mu \text{ (steady state)} \qquad (7)$$

Also, at steady state $dC_s/dt = 0$. Thus, at steady state substituting equation 7 into equation 4 gives:

$$C_x = Y(C_{so} - C_{sf}) \text{ (steady state)} \qquad (8)$$

Assuming that the yield can be treated as a constant, then from equation 4 it is clear that C_x in a continuous stirred tank reactor is proportional to Y, and from equation 5 it is clear that dC_x/dt is proportional to C_x and must therefore be proportional to Y.

Accordingly, the actual value of Y has no effect on dC_s/dt in equation 6 since both terms divided by Y are proportional to Y. A dummy yield of 0.1 g of cells per g of substrate will suffice. Although the assumption of constant

yield may be a source of error in the determination of the kinetics of a population subgroup, it is likely that this error would be smaller than those involved in methods requiring an estimation of the portion of total cell mass belonging to a particular subgroup.

If a slug addition of a substrate for a particular subgroup is given to a continuous stirred tank reactor at steady state, C_{sf} and dC_s/dt can be measured against time. If C_{so} and D are known, the initial value of C_x can be determined from equation 8. Equation 6 can then be used to estimate dC_x/dt and to give a progressive estimate of C_x over short time periods. If the time periods are from 1 to 2 h, the increase in C_x is small compared with total C_x, so that there is only a small error if the initial value of C_x for each time period is used. Equation 5 can be used to determine μ for the time interval, and this can be plotted on a Lineweaver-Burk plot against the average substrate concentration for the time period to give an estimate of μ_{max} and K_s. This method allows a comparison to be made of the kinetics of the predominating organisms in a particular subgroup at different retention times, that is, the desired transient kinetics.

MATERIALS AND METHODS

Medium and growth conditions. The digesters were fed a defined medium consisting of the following (moles per liter): NH_4Cl (2×10^{-2}), KH_2PO_4 (2.2×10^{-3}), K_2HPO_4 (8.6×10^{-4}), $MgCl_2 \cdot 6H_2O$ (5.0×10^{-3}), $CaCl_2$ (5.4×10^{-5}), $FeCl_2 \cdot 4H_2O$ (2×10^{-4}), $MnCl_2 \cdot 4H_2O$ (3.3×10^{-5}), $ZnSO_4 \cdot 7H_2O$ (6.0×10^{-6}), $CuCl_2 \cdot 2H_2O$ (6.0×10^{-7}), $CoCl_2 \cdot 6H_2O$ (8×10^{-6}), $NiSO_4 \cdot 6H_2O$ (2.5×10^{-6}), $Al_2(SO_4)_3 \cdot 18H_2O$ (3.9×10^{-7}), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (4.9×10^{-7}), H_3BO_3 (1.6×10^{-6}), KCl (7.0×10^{-3}), and Na_2S (1×10^{-3}).

Variable amounts of acetate, propionate, butyrate, and glucose were added to this basal medium as required. Medium was fed continuously through peristaltic pumps to two digesters (6.27 liter and 6.55 liter). The medium had been sparged with high-purity nitrogen and was kept under a nitrogen headspace. All digester connections were through butyl rubber tubing, and the digesters were stirred through an airtight mechanical seal at 900 and 1,400 rpm, respectively, with 3-in. (ca. 7.62-cm)-diameter flat blade impellers. The outlet was through an overflow weir and an inverted siphon. The digester was operated as a continuous stirred tank with no recycle. The pH was maintained at 7.0 by automatic titration with 3 M NaOH throughout the experiments, and the temperature was kept at 35°C. The original inoculum was from a municipal sewerage digester.

Propionate production from glucose. Propionate resulting from the breakdown of input glucose was treated as part of the total input propionate concentration, C_{so}, in equation 2. The method described by Boone (2), in which the rate of decay of labeled propionate is compared with the rate of decay of the propionate pool, was used to measure the total rate of propionate decay (Q_sC_x) for a short time during the transient. Equation 2 was then used to calculate C_{so}, which includes propionate fed directly and propionate resulting from glucose degradation. The propionate resulting from glucose degradation could then be obtained by subtracting the concentration of propionate in the medium from the calculated value of C_{so}.

[2-^{14}C]propionate was separated from the samples by injecting 1 ml of sample into a Waters high-performance liquid chromatograph fitted with a refractive index detector and a C18 column. A 4% KH_2PO_4 solution reduced to pH 3.0 with H_3PO_4 was used as the mobile phase. A spike of unlabeled propionate was added so that obvious peaks resulted in all cases as propionate passed through the detector. The fluid leaving the detector was collected in preweighed vials as the propionate peak was registered, and after reweighing, 3 ml of this was mixed with 17 ml of Phasar cocktail. The scintillations were counted for 15 min on a Packard model 3255 scintillation counter. Volatile acids were measured by gas chromatography as described previously (6).

Hydrogen measurement. A butyl rubber stopper was fitted into the top of the digester allowing direct sampling of the gas phase with a 5-ml gas-tight syringe. The gas was injected into a Gow Mac gas chromatograph model 69550 measuring thermal conductivity. This was fitted with a column (5 feet [ca. 152.4 cm]; 0.5 in. [1.27 cm.]; molecular sieve, 5A) and run at ambient temperature. Peak areas were measured by weight and compared with a standard. All samples were measured in duplicate, generally giving a precision of ±5%. Accuracy was ±8%, with the analysis being sensitive to approximately 3.5×10^{-5} atm (3.5×10^{-3} kPa).

RESULTS

To obtain growth kinetics, digesters were allowed to reach steady state at a particular residence time and then given a slug addition of neutralized propionate and [2-^{14}C]propionate. The criteria for steady state was that the digester had been held at the same residence time and pH 7.0 for five residence times and that there was no trend in measured concentrations of volatile acids in the digester or hydrogen in the gas phase. The decay rates of unlabeled and labeled propionate were then measured with time. The kinetic analysis technique described in the introduction was then used to obtain values of μ_{max} and K_s for the subgroup of propionate-using organisms that predominated under the conditions tested. When pH sensitivity was tested, the pH was dropped from 7.0 to 6.0 with HCl as the propionate slugs were added.

The decay rates of labeled and unlabeled propionate for digesters at residence times of 14.5 and 8.2 days at pH 7.0 are shown in Fig. 1, 2, and 3. The relationship between radioactivity and time is not linear (2), and the dotted lines joining the points are not meant to imply a linear relationship. The production of propionate from glucose was 0.34 mg/mg at a retention time of 14.5 days and 0.18 mg/mg at a retention time of 8.2 days. The digester at a retention time of 14.5

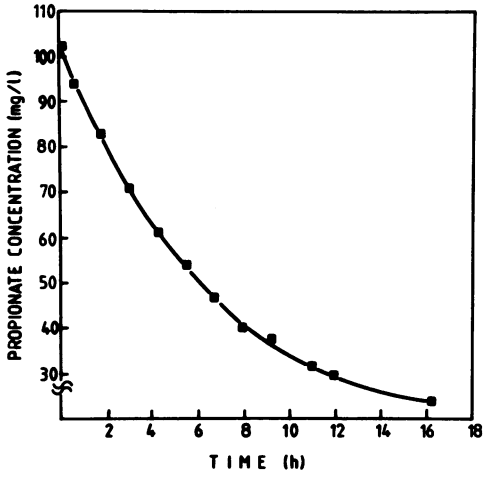

FIG. 1. Propionate decay in digester at a retention time of 8.2 days fed medium A (acetate, 2,330 mg/liter; propionate, 1,030 mg/liter; butyrate, 470 mg/liter).

days had 0.001 M S^{2-} as a sulfur source, whereas the digesters at a retention time of 8.2 days had 0.001 M SO_4^{2-} as the sulfur source. The sulfur, however, only accounted for between 7 and 1.5% of the total hydrogen production, so that the propionate-using organisms were largely relying on methanogens to maintain a low hydrogen concentration. The sulfate concentration was only sufficient to account for approximately 10% of the hydrogen produced from propionate at steady state in both digesters run at a retention time of 8.2 days. Consequently, even if sulfate-reducing, propionate-using organisms such as *D. propionicus* could out-compete other sulfate-reducing organisms for the available sulfate, they would form less than 10% of the propionate-using population and would not be able to increase their substrate uptake rate when additional propionate was added.

The hydrogen concentration did not vary significantly during the course of the individual experiments or as a result of changing the pH from 7.0 to 6.0. As expected, the hydrogen concentrations at the same retention time for digesters fed glucose were the same as for digesters fed only acids, since they were allowed to reach steady state before the experiments commenced. All three experiments at a retention time of 8.2 days had hydrogen concentrations in the range of 0.56×10^{-4} to 0.67×10^{-4} atm (0.57×10^{-2} to 0.68×10^{-2} kPa), whereas at a retention time of 14.5 days, the range was 0.38×10^{-4} to 0.47×10^{-4} atm (0.38×10^{-2} to 0.48×10^{-2} kPa).

The digester run at a retention time of 14.5 days was found to have a μ_{max} of 0.0054 h^{-1} and a K_s of 11 mg/liter. The digester run at a retention time of 8.2 days without glucose had a μ_{max} of 0.059 h^{-1} and a K_s of 250 mg/liter, whereas the digester run at a retention time of 8.2 days with glucose had a μ_{max} of 0.040 h^{-1} and a K_s of 410 mg/liter. It is clear that the kinetic constants for the propionate users at a retention time of 8.2 days are an order of magnitude different than those at a retention time of 14.5 days, indicating that two distinct subgroups of propionate users are involved. This difference cannot be attributed to the differences in hydrogen concentration since the faster growing subgroup was grown at the higher hydrogen concentration. The two results for the retention time of 8.2 days can be taken as an indication of the repeatability of the analysis, giving average values of 0.050 h^{-1} for μ_{max} and 330 mg/liter for K_s.

The response to a slug addition of propionate in conjunction with a pH shock to pH 6.0 (Fig. 4) gives additional evidence for the existence of two distinct groups of propionate users. For the digester run at a retention time of 14.5 days, the propionate production from glucose was also measured showing that 0.031 mg/mg was produced. The digester run at a retention time of 14.5 days was strongly inhibited by the pH shock. The added propionate failed to decay, despite the fact that the propionate production from glucose was only about 10% of the amount produced at pH 7.0. Neither hydrogen concentration nor acetate concentrations (unpublished data) increased with the change in pH, so that the observed inhibition of propionate users cannot be attributed to end-product inhibition.

The pH shock given to the digester at a retention time of 8.2 days was conducted 1 day after the growth rate at pH 7.0 was measured. It would therefore be expected that C_x would be slightly higher than the C_x value at steady state and that calculated values of μ using the steady-state value of C_x would be slightly higher than expected if the propionate organisms were uninhibited. Table 1 compares values of μ measured

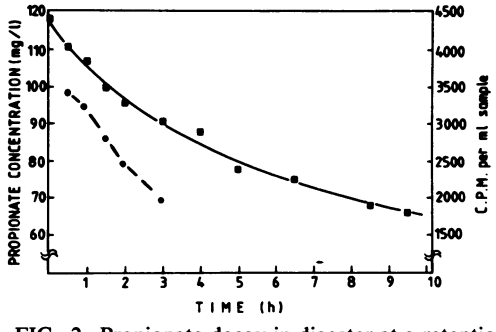

FIG. 2. Propionate decay in digester at a retention time of 8.2 days (■) and counts per minute of [2-^{14}C]propionate per milliliter of sample (●) fed medium B (acetate, 270 mg/liter; propionate, 985 mg/liter; butyrate, 225 mg/liter; glucose, 10,130 mg/liter).

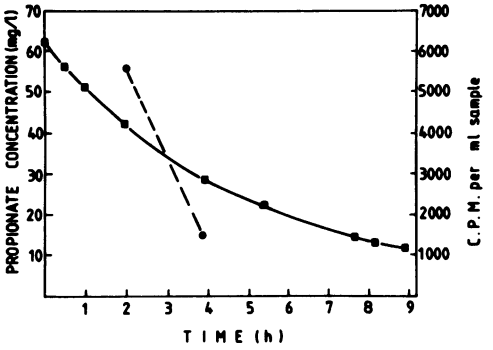

FIG. 3. Propionate decay in digester at a retention time of 14.5 days (■) and counts per minute of [2-^{14}C]propionate per milliliter of sample (●) fed medium C (acetate, 310 mg/liter; propionate, 2,550 mg/liter; butyrate, 245 mg/liter; glucose, 9,840 mg/liter).

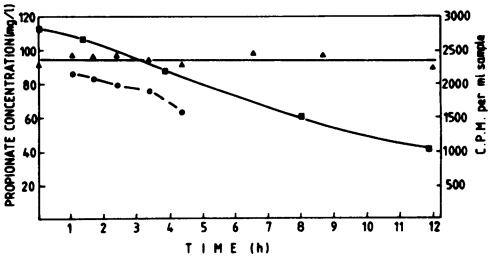

FIG. 4. Propionate decay at pH 6.0 in digester at a retention time of 8.2 days fed medium A (■), propionate decay at pH 6.0 in digester at a retention time of 14.5 days fed medium C (▲), and counts per minute of [2-^{14}C]propionate per milliliter of sample at a retention time of 14.5 days (●).

in this experiment (pH 6.0) with the values of μ predicted from the kinetics of cells grown at pH 7.0. At this retention time, the predominating propionate users show a slight inhibition for the first hour followed by a recovery to growth rates expected of an uninhibited culture.

DISCUSSION

There appears to be at least two major subgroups of propionate-using organisms that can predominate in mixed-culture anaerobic digesters with low sulfate concentrations, depending on the retention time of the digester. Organisms predominating at a retention of 14.5 days without sulfate were found to have a μ_{max} of 0.0054 h^{-1} and a K_s of 11 mg/liter, which is similar to the reported maximum specific growth rate of *S. wolinii* grown in coculture with a methanogen of 0.004 h^{-1} (3). This is also similar to a K_s value of 7 mg/liter reported for propionate users in mixed sludge from a waste treatment plant (8), although the retention time is not reported in this case.

The second subgroup, predominating at a retention time of 8.2 days and relying on methanogens to use at least 93% of the total hydrogen produced, had entirely different kinetics with an average measured μ_{max} of 0.050 h^{-1} and K_s of 330 mg/liter. The kinetic constants reported by Lawrence and McCarty (9), with a μ_{max} of 0.017 h^{-1} and K_s of 32 mg/liter, lie between the kinetics measured for these two groups. It appears that the method used by Lawrence and McCarty, which assumed that there was only one group of propionate users and collected data over a wide range of retention times, was responsible for obtaining constants that could not be related to either group.

The inclusion of a decay term (K_d) as used by Lawrence and McCarty (9) does not significantly affect the results. The actual growth rate is the observed growth rate plus the decay rate. In the case of the digester run at a retention time of 14.5 days, inclusion of a decay term increases the estimate of μ_{max} from 0.0054 to 0.0060 h^{-1} (using a decay rate of 0.015 day^{-1} or 0.00063 h^{-1}). For the digester run at a retention time of 8.2 days, the estimate of μ_{max} increases from 0.050 to 0.051 h^{-1}. These differences are small compared with differences between the two kinetic analyses at a retention time of 8.2 days (0.040 and 0.059 h^{-1}).

By incorporating the constants obtained into the standard Michaelis-Menten expression and calculating the residual substrate levels expected for retention times of 8.2 and 14.5 days, it can be seen how retention time selects the predominating group. At a retention time of 14.5 days, the slow-growing propionate users should give a residual substrate level of 12 mg/liter, whereas the fast-growing propionate users should give a residual substrate level of 20 mg/liter. At a retention time of 8.2 days, the slow-growing propionate users give a residual substrate level of 175 mg/liter, whereas the fast-growing propionate users give a residual substrate level of 37 mg/liter. In both cases, the group selected was the group able to bring the residual substrate level to the lower concentration.

The two groups varied markedly in their response to a pH change from 7.0 to 6.0. The slow-

TABLE 1. Response to shock from pH 7.0 to 6.0 in digester run at a retention time of 8.2 days

Time after pH shock (h)	Average propionate concn (mg/liter)	Actual μ (h^{-1})	Predicted μ if uninhibited (h^{-1})	Difference (%)
0–1.3	110	0.0085	0.0125	−47
1.3–3.7	98	0.012	0.011	+8
3.7–7.9	75	0.010	0.0093	+7
7.9–11.7	52	0.0088	0.0068	+23

growing group was very sensitive to pH change, whereas the faster-growing group was less sensitive. This provides additional evidence for the existence of two subgroups of propionate users and is of importance in predicting the effect of pH changes in working digesters. Digesters that select for the faster-growing propionate users will be less sensitive to pH shock with respect to degradation of propionate.

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