Kinetic measurements on bacterial cultures growing on fibres

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The culture outflow-rate from a specially designed pH-auxostat continuous culture system can be used to calculate the specific growth rates of different bacterial species on suspensions of cellulose or other insoluble substrates. Provided a constant concentration of substrate is maintained in the inflowing medium and the contents of the culture vessel are well mixed, steady-state conditions are established which permit the determination of the rate, per unit volume of culture, and extent of solubilization of the substrate under defined environmental conditions. Preliminary results of kinetic measurements on cultures of four species of fibrolytic rumen bacteria, growing on finely dispersed cellulose, are presented.

Die invloeitempo van kultuur uit 'n spesiaal ontwerpte pH-ouksostaat deurlopende kultuursisteem kan gebruik word om die soortlike groeitempo's van verskillende bakteriesoorte op sellulose suspensies of ander onoplosbare substrate te bepaal. Indien die substraatkonsentrasie in die inkomende medium konstant gehou word en die inhoud van die kultuurhouer deeglik gemeng word, ontwikkel bestendige-staat toestande wat die bepaling van die tempo, per eenheidvolume kultuur, en die omvang van oplosbaarmaking van die substraat onder gedefinieerde omgewingstoestande moontlik maak. Die voorlopige resultate van kinetiese metings op kulture van vier soorte veselverterende rumenbakterieë, wat op fyn verdeelde sellulose gekweek is, word aangebied.

Keywords: Cellulose, continuous culture, fibre, pH-auxostat, rumen bacteria, solubilization, specific growth rate

Introduction

Much information is available on the extent of solubilization of intact plant cell walls and their isolated main constituents, cellulose and hemicellulose, on extended incubation with cultures of individual species of fibrolytic rumen bacteria or suspensions of the mixed microbial population of the rumen. In contrast, very little has been published on the specific growth rates of these species on plant fibres of different origin, untreated or treated, and the effect of environmental factors such as pH on the growth rates. The reason for this is the difficulty in measuring growth rates on insoluble substrates. Most methods for the determination of specific growth rates depend on repeated measurements of biomass concentration or cell numbers. This applies not only to batch culture procedures, but also to determinations in chemostat cultures by the gradual approach to critical dilution rate, or by the rate-of-washout method. The strong adhesion of most fibrolytic bacterial species from the rumen to their insoluble substrates interferes with the precise determination of numbers, biomass, or culture properties proportional to biomass.

A possible way of overcoming these technical problems was to grow the micro-organisms in pH-auxostat continuous culture (Martin & Hempfling, 1976) in a weakly buffered medium, in which the insoluble substrate was evenly dispersed, and calculate the specific growth rates. In addition, once steady-state conditions were attained, the extent and rate of solubilization of the substrate under the given environmental conditions could be calculated from the difference in substrate concentration between influent medium and effluent culture.

The present communication describes briefly the apparatus and procedures used and presents preliminary results of kinetic measurements on four species of fibrolytic rumen bacteria growing on finely dispersed cellulose.

Materials and Methods

Organisms

Bacteroides succinogenes S85 and *Ruminococcus flavefaciens* FD1 were kindly donated by Dr Marvin P. Bryant in about 1960. *Ruminococcus albus* 22.08.6A and *Clostridium polysaccharolyticum* B came from the culture collection of this laboratory.

Medium

Medium No. 10 of Caldwell & Bryant (1966) was modified by substitution of 0,1% ball-milled Whatman No. 1 filter paper, washed free of soluble degradation products, for the mixture of carbohydrates. The stock mixture of volatile fatty acids was adjusted to pH 7,5; the Na_2CO_3 concentration lowered to 6,6 mM and the medium equilibrated with an oxygen-free gas mixture of 5% H_2 + 10% CO_2 in N_2. The pH of this medium at 39° was 7,0.

Apparatus

From the work of Gray (1978) it was evident that, with the use of conventional culture apparatus, problems could be expected with the maintenance of a constant substrate concentration in the inflowing medium. Figure 1 is a simplified presentation of the special form of pH-auxostat continuous culture system which was evolved after much experimentation to overcome these difficulties.

The growth-linked changes in the pH of the culture in the water-jacketted vessel (1) were detected by the single-probe pH electrode (2) and caused a pH-controller to activate the electro-pneumatically actuated valve (3) to add weakly buffered medium of a higher pH-value from reservoir (4) until the set value was restored. The hold-up volume of the narrow-bore medium line and valve was less than 1 ml, thus minimizing fluctuations in substrate concentration in the influent medium as a result of sedimentation. As exponential growth occurred, a regular pattern of medium addition became established. Excess culture was displaced *via* the exit port into harvest receiver (5), at a rate equal to that of inflow of fresh medium, this process being aided by a slow flow of sterile, oxygen-free gas mixture (5% H_2, 10% CO_2, balance N_2) through the head space of the culture vessel. The harvest receiver was suspended from a sensitive load cell (type SM-50, Interface Inc., Scottsdale, USA), the output of which was recorded by a stripchart recorder. Oscillatory mixers (6) ('Vibromixer' type E1, Chemap AG., Männedorf, Switzerland), with non-rotating shafts passing through elastomer disc seals in the entry ports, ensured efficient dispersion of the substrate in the medium reservoir and the culture vessel. An incubation temperature of 39° was maintained by the circulation of water from a constant temperature bath through the jacket of the culture vessel.

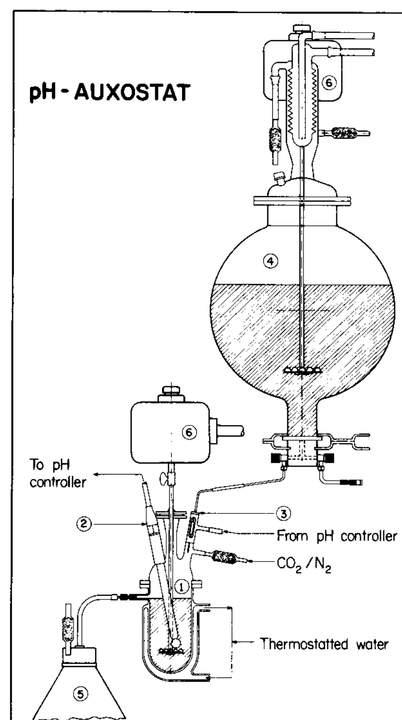

Figure 1 Simplified presentation of pH-auxostat for kinetic measurements on bacterial cultures growing on fibres. Refer to text for a description of the apparatus.

Determination of specific growth rates

The mean hourly output of culture on attainment of steady-state conditions was calculated from the straight-line mass *vs* time tracing on the recorder chart and the density of the culture medium. The specific growth rate, equal to the dilution rate (D), was calculated from the ratio of output rate of culture to working volume of the culture vessel.

Determination of rate and extent of solubilization of cellulose

The concentrations of cellulose in the medium reservoir (s_r) and in the culture at steady-state ($\bar{s}$) were determined by the phenol-sulphuric acid method. These were used in the following calculations:

Rate of solubilization (mg $l^{-1}h^{-1}$) $= D(s_r - \bar{s})$

Extent of solubilization (%) $= 100(s_r - \bar{s})/s_r$

Test for constancy of s_r

Starting with a full reservoir, a 5 ml sample of medium was drawn from a sampling point branching off from the medium outlet port, followed by a 1 litre portion which was saved for re-use. This procedure was repeated until the medium reservoir was empty. Triplicate estimations of cellulose were run on each of the 12 samples collected in this way.

Results and Discussion

Constancy of substrate concentration in the inflowing medium

The mean concentration of cellulose in the 12 samples of medium from the reservoir was 0,95 mg ml^{-1} (expected: 1,0 mg ml^{-1}), and the coefficient of variation between samples was 3,1%. This was considered satisfactory for a valid comparison of cellulose concentrations in the medium and in the cultures at steady-state.

Growth in the pH-auxostat

In repeated runs, the initial batch cultures of *Bacteroides succinogenes* S85 in the pH-auxostat showed growth only after a lag of several days. When the pH-controller was activated, the measured growth rates increased progressively (*e.g.* from 0,16–0,36 h^{-1} over a period of two days) before levelling out. Whereas the cells, even in 16 h cultures, in cellobiose-rumen fluid medium, were generally large and very pleomorphic, those in the cellulose medium were uniformly coccoid to lemon-shaped, about 0,4–0,5 µm in diameter, with the great majority of cells attached to fibres. Notwithstanding reports about the fragility of the cell walls of *B. succinogenes*, SEM photomicrographs showed no evidence of rupturing of cells by the vigorous agitation in the pH-auxostat. *Ruminococcus flavefaciens* usually became established within about 20 h after inoculation and progressed rapidly to relatively high specific growth rates. The residual fibres were densely colonized by cells of about 0,4 µm in diameter, commonly arranged in chains. *Ruminococcus albus* 22.08.6A, on the other hand, required several days to reach a constant specific growth rate. The cells were strongly attached to the residual fibres, but the density of colonization was never as high as with *R. flavefaciens* FD1. *Clostridium polysaccharolyticum* B was established in continuous culture with variable success. Occasionally, repeated inoculation of the medium in the pH-auxostat failed to initiate growth, while at other times high growth rates were attained in less than one day. The cells did not appear to attach to the cellulose to any degree, as was earlier reported by Morris & Van Gylswyk (1980). The cultures became quite viscous and the vibratory stirrer had to be run at full voltage to obtain adequate mixing.

Specific growth rates; rates and extents of solubilization of cellulose

Table 1 shows preliminary results of determinations on one strain each of four species of fibrolytic rumen bacteria. The pH of the incoming medium was 7,0 while the pH-controller operated at pH 6,88 ± 0,03. The growth rates found were unexpectedly high, while the extents of solubilization of Whatman No.1 filter paper were much lower than those reported for batch cultures of the same species (and, in some cases, the same strains) incubated for 14 days (Morris & Van Gylswyk, 1980). Both observations are probably related to the operating conditions chosen for our initial experiments. With the poorly buffered medium used and the small pH differential between medium and culture, a low degree of solubilization and fermentation of the substrate produced sufficient acid to lower the pH of the medium to the set point of the controller, causing the addition of fresh medium. Presumably, in this way the more readily degraded regions of the cellulose particles were never depleted and the growth rates of the organisms were not limited by the rates at which they were able to degrade the more refractory portions of the substrate. This would explain both the low extents of cellulose solubilization and the relatively high growth rates found. Indications have since been obtained

Table 1 Specific growth rates of, and extents and rates of solubilization of filter paper cellulose by, four species of fibrolytic rumen bacteria

Species and strain	Specific growth rate (h^{-1})	Solubilization of cellulose: Extent (%)	Solubilization of cellulose: Rate (mg $l^{-1}h^{-1}$)
Bacteroides succinogenes S85	0,38	n.d.	n.d.
Ruminococcus flavefaciens FD1	0,51	21	110
Ruminococcus albus 22.08.6A	0,20	21	43
Clostridium polysaccharolyticum B	0,59	18	129

n.d. — not determined.

that, on leaving the pH in the culture vessel unaltered, but increasing that of the medium in the reservoir stepwise, the extent of solubilization by the cultures increases progressively. Eventually the point is reached where the substrate is depleted, or it cannot be further degraded by the bacterial species concerned, before sufficient acid is produced to trigger the addition of further medium. Growth then comes to a standstill. At the same time, this stepwise widening of the pH differential between medium and culture appears to depress the growth rates of the individual species to different extents. This aspect is being examined in greater detail at present. The rates of cellulose solubilization shown in Table 1 fall far short of those occurring in the rumen. Even taking the highest value, this would only account for the digestion of 31 grams per day in a sheep with 10 l rumen contents, whereas one would expect almost tenfold this amount. However, it should be pointed out that cellulose solubilization could be depressed in two ways. First, filter paper cellulose is probably more highly ordered and therefore more difficult to degrade than the form of cellulose present in plant cell walls. Secondly, the use of monocultures eliminates synergistic interactions which are known to occur between different species in the rumen.

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