The Continuous Culture of Anaerobic Bacteria

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(Accepted for publication 9 November)

SUMMARY

Modifications to an anaerobic continuous culture apparatus to allow pH control, and pH and Eh measurements, are described. Two anaerobic rumen bacteria were grown under different conditions, but as carbohydrate-limited cultures. The effects of growth rate, pH value and Eh value on yields of bacteria, enzyme activities and fermentation products are described. Optimum bacterial yields per mole of substrate fermented and per mole of ATP presumably formed in the fermentation were variable with the particular bacterium and the substrate, and were high for hexose fermentations. Yields of bacteria varied with growth rate, being lowest at low growth rates. Fermentation products also varied with growth rate and the pH value of the culture in some cases, as did the production of enzymes. Maximum growth rates calculated from batch cultures were in agreement with those found in the continuous cultures.

INTRODUCTION

Some results obtained from continuous cultures of anaerobic rumen bacteria were described by Hobson (1965*a*). We give here some further observations on continuous cultivation of anaerobic bacteria in a chemostat with external pH control.

METHODS

Apparatus. The apparatus described by Hobson (1965*b*) relied for pH control on the buffering action of the medium, but it was mentioned that external pH control had been incorporated in a later modification of the apparatus. The present apparatus, which has been in use for some years, is basically the same as that previously described (Hobson, 1965*b*) with the following modifications (reference is made to Fig. 1, Hobson, 1965*b*, in this description.) The single gas flow-meter (P) has been replaced by a double flow-meter on the 'apparatus' side of the furnace. This enables not only the oxygen-free carbon dioxide from the furnace to be monitored, but also a stream of some other gas, such as oxygen, to be introduced into the gas flow. In place of the balloon A a carbon-dioxide line is taken from the junction N to the filter at A to replace medium in flask B by carbon dioxide as the flask is emptied. This lead is clipped off close to A when the flask is to be changed, to obviate entry of air into the tubing. The culture vessel F has been replaced by one of similar design but of 250 ml. working volume, and the stirring gas flow increased to 250 ml./min. The rubber bung forming the top of the vessel is now as follows. A large diameter hole is bored centrally through the rubber bung and through this is inserted a combination glass-calomel electrode (E.I.L., type SHDN33. Electronic Instruments Ltd., Richmond, Surrey). This has a rubber sleeve part-way up it which, greased with silicone grease, makes the electrode a gas-tight fit

in the hole and allows the electrode to be inserted without damage to the glass tip. Arranged around this are the inlet and sampling tubes shown in the original diagram and also inlets for alkali and acid. These latter are stainless steel tubes of $\frac{1}{8}$ inch (0.32 cm.) outside diameter to the projecting tops of which are soldered steel tubes of larger diameter into which fit smaller tubes in the manner of a cone and socket joint. Also inserted through a further hole so that the silicone-greased ebonite cap is about $\frac{1}{4}$ inch (1.27 cm.) into the bung is an E.I.L. platinum electrode type EPT 23. The steel tubing joints on the acid and alkali inlets are connected to small bore glass tubes which pass via stopcocks to rubber tubing which can be opened or closed by a Pye titrator delivery unit (Pye Instruments, Cambridge) adjusted so that each solenoid opens to the same extent. The upper ends of these tubes are connected to glass joints and then by glass tubing to alkali and acid reservoirs at a height of about 18 inches (45.7 cm.) above the culture vessel. Sterile air is admitted to these reservoirs through cottonwool plugs and 'Carbosorb'-containing tubes. The platinum electrode circuit is a recent addition and was not used in all the experiments reported here, and may be omitted. The glass electrode is connected to an E.I.L. pH meter/controller model 91B, and the platinum electrode to a similar instrument graduated in millivolts (this is at present used only as a meter). The calomel reference electrode can be connected by means of a switch to either the pH meter or the millivoltmeter in circuit with either the glass or platinum electrode. The pH meter is also connected to a 'Dwarf' recorder (Everett-Edgcumbe Ltd., Colindeep Lane, London), and via the upper and lower controller circuits to the solenoids actuating the inflow of acid or alkali. In use the lower indicator of the pH meter/controller is set at the appropriate pH value. In most cases control necessitates only the addition of alkali as fermentation tends to lower the pH value of the culture medium (from its initial value pH 6.8) and the upper indicator is set about 0.2 units higher to act mainly as a safety device. The taps on the alkali and acid lines are then set by trial so that a very small amount of alkali is added at each opening of the solenoid valve and overshoot of pH is negligible. When an Eh measurement is to be made the pH meter is returned to the check point (after moving the upper control setting if necessary), the reference electrode is switched to the millivoltmeter and this is then set to the 'read' position. A stable Eh reading is usually obtained in about 5 min. and during this time the pH of the culture does not alter appreciably. To resume pH control the procedure is then reversed. This circuit was adopted so that a separate reference electrode need not be inserted in the culture vessel, but if the Eh value were to be continually recorded then a second electrode would be needed. No 'poisoning' of the platinum electrode by culture constituents has been noted in runs lasting many hundreds of hours.

Originally a pH controller working by mechanical contacts on a 'slave' meter operated by a Pye pH meter was used in the circuit, but the contacts on this were liable to stick in the 'on' position and breakdowns were comparatively frequent. The E.I.L. meter/controller is much more compact and reliable, and extremely stable.

All the parts of the apparatus including the solenoid valve tubings and the acid and alkali reservoirs and the acid and alkali solutions are sterilized by autoclaving (120°, 25 min.) and assembled aseptically. The two electrodes are sterilized by immersion for 24 hr in a solution of 1% (v/v) H_2SO_4 in 70% (v/v) ethanol in water. This treatment has had no adverse effects on the electrodes, and no contamination of the cultures attributable to the electrodes has occurred. The pH meter is initially

standardized with a second electrode system and external buffers, the culture electrode is then connected up and the pH of the uninoculated medium determined. This can then be checked against the pH value of a sample of the medium measured on an external pH meter. The check point of the pH meter is noted at about weekly intervals during a run, but usually drift amounts to only about 0.1 pH units per fortnight.

In some chemostat pH control systems (e.g. Wright, 1960) a timer is used to add the acid or alkali at short intervals during the 'on' cycle. With the taps in the alkali/acid addition circuit as described here the rate of addition of the solutions (3% NaOH, w/v, or H₂SO₄, v/v) can be adjusted so that overshoot is negligible and our pH control is to better than 0.1 unit. Some adjustment of the relative positions of the acid and alkali inlets and the glass electrode may be needed to get optimum control.

A difficulty of growing the anaerobic bacteria used in the present work is the diffusion of air through the rubber tubing used in the medium circuits. This is negligible with some rubbers, but with other rubber tubings we have found that although air diffusion may not oxidize the medium to the point where a trace of the pink resazurin colour is seen, it can still increase the Eh value sufficiently to stop the growth of some organisms. Since rubber tubings, even from the same supplier, seem to vary from batch to batch we have not found any that can be unequivocally recommended. Neoprene is the best we have so far used, but this may split on autoclaving. Hungate (1963) and Hungate, Smith & Clarke (1966) have also considered the problem of diffusion of oxygen through rubber in anaerobic cultures. The latter authors recommend butyl rubber stoppers for culture tubes. The wall thickness of the tubing should be as great as possible consistent with flexibility. Although much of the tubing may be of glass or stainless steel some rubber is needed, in parts, to give flexibility to take up strains and vibrations in the apparatus (and during autoclaving) and to allow joints to be connected.

Bacteria. The lipolytic Bacterium 5S was the strain previously used and described by Hobson (1965*a*). Two strains of *Bacteroides amylophilus* were isolated by our colleague Dr T. H. Blackburn and were similar to the strains of *B. amylophilus* described by Blackburn & Hobson (1962). This bacterium ferments only starch, dextrans or maltose and is one of the more important proteolytic bacteria in the rumen, producing amylase and protease, both excreted into the culture medium.

Media. The medium for growth of bacterium 5S on glycerol in an atmosphere of 100% CO₂ was described by Hobson (1965*a*). For fructose growth the glycerol was replaced by fructose. When 95% (v/v) N₂ + 5% (v/v) CO₂ was used as atmosphere the sodium bicarbonate concentration in the medium was decreased to 0.02% (w/v), and the cysteine hydrochloride solution brought to pH 6.6 with sodium hydroxide solution before the bicarbonate and carbohydrate were added and the solution filter-sterilized. In batch cultures under this atmosphere M/60 phosphate buffer (pH 6.6), was included to help to control the culture pH value. For incubations under an atmosphere of 100% N₂ bicarbonate was omitted from this medium. The medium for batch and continuous culture growth of *Bacteroides amylophilus* (formulated by Dr T. H. Blackburn to give optimum growth in batch cultures) contained, per 100 ml.; mineral solutions *a* and *b* (Hobson, 1965*a*), 15 ml. each; Bacto tryptose, 0.1 g.; cysteine hydrochloride, 0.1 g.; sodium bicarbonate, 0.6 g.; resazurin, 0.1 ml. of 0.1% solution; maltose hydrate (B.D.H.), to concentration required; water to 100 ml. Stock cultures were kept on slopes of this medium solidified with 2% agar. This bacterium will grow

in a medium containing ammonia as sole nitrogen source, but the addition of tryptose to the medium appeared to decrease the lag phase in batch cultures, although no utilization could be shown (Blackburn, 1965).

General methods of preparing the media and of setting up and inoculating the continuous culture apparatus have been previously described (Hobson, 1965*a*), except that in the later work with *Bacteroides amylophilus* the maltose, bicarbonate and cysteine were added as concentrated filter- or heat-sterilized separate solutions and not as a mixed filter-sterilized solution.

Analytical methods. Methods of sampling the cultures and of determining dry weights of bacteria, glycerol, total volatile fatty acids (VFA), lactic acid and culture turbidity were as previously described (Hobson, 1965*a*). Succinic acid was determined by a manometric method (Umbreit, Burris & Stauffer, 1957). Fructose was determined by the method of Roe (1934) and maltose by the Somogyi-Nelson method (Nelson, 1944). Volatile fatty acids were separated by gas chromatography (on an instrument made by Gas Chromatography Ltd., Maidenhead) by our colleague Mr T. Walker. Bacterial-nitrogen was determined by a Kjeldahl method on bacteria centrifuged down from the culture at 0° and washed once with water at 0°. Total medium-nitrogen was determined by a Kjeldahl method and ammonia-nitrogen by a microdiffusion method. Lipase and esterase activities were determined by using naphthylaurate or acetate as substrates at pH 6.8 (Hobson & Summers, 1966). Protease activity was determined by a modification of the method of Anson (1938) as used by Blackburn (1965), with casein as substrate. Amylase activity was determined by a modification of the method of Walker & Campbell (1963), again as used by Blackburn (1965). Total counts of *Bacterium* 5S were made on a Coulter Counter (Coulter Electronics Ltd., Dunstable, England) with a 30 μ orifice tube at an aperture current setting of 8 and a threshold value of 20 after suitable dilution of the culture in 0.9% (w/v) NaCl solution. *Bacteroides amylophilus* was counted at a threshold value of 35. In all cultures, batch and continuous, the culture turbidity was found to have a linear relationship to bacterial dry weight (mg./ml.) except at very low values of turbidity (about 3 turbidity units) and turbidity was used to monitor the continuous cultures. However, nearly all values of bacterial yields were calculated from actual dry weight measurements and not from turbidity readings.

Growth in the basal media, without carbohydrate, was so small that no accurate estimate of bacterial concentration either by weight or turbidity could be made and this degree of basal growth was neglected in calculating yields.

The definitions of bacterial yields, $Y_{\text{substrate}}$ and Y_{ATP} , are as proposed by Bauchop & Elsdon (1960) and as used in the previous paper (Hobson, 1965*a*).

In all cases there were slight variations in substrate concentrations in the different lots of medium used during each continuous culture run and in different cultures. The nominal concentration of sugar is given, but values for Y and fermentation products are calculated on the analysis of the medium flowing through the culture at any particular time.

The rate of alkali addition to the cultures at pH values about 6–6.5 was small, about 5% of the medium flow rate; dilution rates are given as the actual medium flow rate.

All cultures were incubated at 39°.

RESULTS

Lipolytic Bacterium 5S

Culture on glycerol. Batch cultures showed that glycerol-limited cultures could be run at a glycerol concentration of $59.7 \mu\text{mole/ml}$. (Hobson, 1965*a*); Fig. 1 shows some results from a continuous culture of 1660 hr duration at this glycerol concentration under an atmosphere of CO_2 . The pH value was controlled at pH 6.1 and, as in all the cultures described, at least 30 hr were allowed for the cultures to stabilize at a particular growth rate before measurements were begun. The optimum dilution rate (D) shown is about 0.2 hr^{-1} , but when the medium flow was held at $D 0.19 \text{ hr}^{-1}$ and the pH value decreased to 5.5 the culture rapidly washed out. At this dilution rate (0.19 hr^{-1}) at

Fig. 1

Fig. 2

Fig. 1. Culture of *Bacterium 5S* on glycerol. Culture turbidity ●. Glycerol used ○. Results from one culture of 1660 hr duration. A turbidity of 20 is equivalent to $0.79 \text{ mg. dry wt bacteria/ml}$. D in all figures = dilution rate.

Fig. 2. Culture of *Bacterium 5S* on glycerol. Volatile fatty acid (VFA) production. Results from culture shown in Fig. 1.

pH values above 7 the culture slowly washed out and at pH 7.4 when the dilution rate was changed to 0.21 hr^{-1} a rapid washout took place. Over the range pH 6 to 7 at $D 0.2 \text{ hr}^{-1}$ no differences in bacterial yield were found. Lipase and esterase activities were measured at different growth rates and two peaks of activity (coincident for lipase and esterase) in both bacteria and supernatant fluid were found at dilution rates of about 0.24 hr^{-1} and 0.1 hr^{-1} . The lipase activity of the bacteria showed a maximum at pH 6.6 when tested at four culture pH values. This organism produced acetic, propionic and succinic acids and a little lactic acid when fermenting glycerol (Hobson, 1965*a*); a change in the proportion of the volatile fatty acids with growth rate is shown in Fig. 2. There was a suggestion of a small increase in amounts of fatty acids formed as the culture pH changed from 6 to 7 at $D 0.2 \text{ hr}^{-1}$.

Batch cultures with different concentrations of glycerol were incubated under atmospheres of oxygen-free CO_2 , $\text{N}_2 + \text{CO}_2$ (95+5) and N_2 . At a glycerol concentra-

tion of $28.3 \mu\text{moles/ml.}$ growth was equally rapid in all media and 98% of the glycerol was utilized. However, at glycerol concentrations above this, lack of buffering capacity in the media under $\text{N}_2 + \text{CO}_2$ or N_2 caused a decrease to about pH 5.6, when growth ceased before all the glycerol was utilized. In the medium under 100% CO_2 glycerol up to $76 \mu\text{moles/ml.}$ was utilized. The yield of bacteria per mole of glycerol fermented was similar in all cultures (about 17). These results showed that high concentrations of carbon dioxide were not essential for growth of the bacterium 5S, so continuous cultures were conducted with the medium as before but with the sodium bicarbonate concentration decreased to 0.02% (w/v) and with a gas phase $\text{N}_2 + \text{CO}_2$ (95+5). The results of two cultures of 300 and 400 hr duration are combined in Fig. 3. One culture

Fig. 3

Fig. 4

Fig. 3. Culture of Bacterium 5S on glycerol under an atmosphere of $\text{N}_2 + \text{CO}_2$ (95+5 by vol.). Results from two cultures of 300 hr and 400 hr duration. A turbidity of 20 is equivalent to 0.90 mg. dry wt bacteria/ml.

Fig. 4. Culture of Bacterium 5S on fructose. Culture turbidity O. Fructose used ●. Results from a culture of 1150 hr duration. A turbidity of 20 is equivalent to 0.92 mg. dry wt bacteria/ml.

was controlled at pH 6.2 and the other at pH 6.5. When the culture was changed to pH 5.4 at $D 0.12 \text{ hr}^{-1}$ a rapid washout occurred. In the culture at pH 6.5 the values of Y_{glycerol} were, at $D 0.057 \text{ hr}^{-1}$, 15.8; $D 0.092 \text{ hr}^{-1}$, 16.1; $D 0.128 \text{ hr}^{-1}$, 20.2, and volatile fatty acids produced were over 90/100 μmoles glycerol used. Glycerol used was 97% of that added at dilution rates about 0.1 hr^{-1} .

Culture on fructose. Batch cultures containing different concentrations of fructose showed that the growth of Bacterium 5S was proportional to fructose used at concentrations of fructose up to $30.6 \mu\text{moles/ml.}$ Above this concentration pH changes stopped growth before all the fructose was utilized. The fermentation products were in all cases acetic and propionic acids at a total of $200 \mu\text{moles/100} \mu\text{moles}$ fructose used and in the amounts $55 \mu\text{moles}$ acetic acid and $145 \mu\text{moles}$ propionic acid. Growth between 18 and

24 hr of incubation was logarithmic. A value for growth rate between these times was calculated by using the equation

$$\mu t = 2.303 (\log n - \log n_0),$$

where n and n_0 were determined from the graph of culture turbidity against total count (see below). With increasing initial concentration of fructose (nominally 0.1, 0.2, 0.4, 1.0, 2.0%, w/v) values for μ of 0.108, 0.123, 0.125, 0.167, 0.161 hr⁻¹ were obtained.

Continuous cultures of *Bacterium* 5S were run at a fructose concentration of 27.8 μ moles/ml. The results from a culture of 1150 hr duration at pH 6.2 are shown in Fig. 4. Total counts were made on a number of samples from this culture; Fig. 5 shows that total bacterial numbers were in linear relationship to bacterial mass con-

Fig. 5

Fig. 5. Culture of *Bacterium* 5S on fructose. Total count ($\times 10^{-9}$) = 0.42 turbidity - 2.7. Culture turbidity $\circ$. Total count ($\times 10^{-9}$) = 7.31 dry bacteria - 1.0. Bacterial dry weight $\bullet$.

Fig. 6

Fig. 6. Culture of *Bacteroides amylophilus* on maltose. Results from a culture of 890 hr duration. A turbidity of 14 is equivalent to 0.9 mg. dry wt bacteria/ml. At points indicated $\bullet$ a true steady state was not obtained (see text).

centration and to culture turbidity. The bacterial yield (Y_{fructose}) increased to a maximum of 60 (average of four determinations) at dilution rates about 0.1 hr⁻¹. The fermentation products were acetic and propionic acids in total amounts about 200 μ moles/100 μ moles fructose used at all dilution rates. No difference was found in culture turbidity at culture pH values of 6.2 and 6.5 at D 0.08 hr⁻¹. At D 0.1 hr⁻¹ changing the culture pH to 5.6 or 7.0 resulted in a slow washout of the cells.

Bacteroides amylophilus

Culture on maltose. Batch cultures of *Bacteroides amylophilus* at different maltose concentrations showed that growth was proportional to maltose used at concentrations up to 11.7 μ moles/ml. and above this concentration low pH values or lack of nitrogen limited the growth. The amount of maltose left at the end of the log phase of growth was similar in each culture and was about 0.82 μ moles/ml. When growth ceased lysis

of the organisms was rapid and the culture turbidity decreased. Acetic, formic and succinic acids were formed. The total volatile fatty acids formed during the log phase of growth at all concentrations of maltose were similar and averaged $138 \mu\text{moles}/100 \mu\text{moles maltose used}$; succinic acid was not determined quantitatively. The yields of bacteria per mole of maltose used (Y_{maltose}) during the log phase of growth at different initial maltose concentrations from 2.04 to $11.7 \mu\text{moles/ml.}$ were: 110, 102, 87, 78, 77, respectively. Since the culture turbidity was small for this bacterium, *B. amylophilus*, an accurate determination of total count from culture turbidity was not possible at low concentrations of maltose in the batch cultures, but at higher concentrations where the culture turbidity corresponding to n_0 was higher, values for total counts and hence growth rates could be determined. These suggested a maximum value of μ of about 0.46 hr^{-1} .

Continuous cultures were run at a maltose concentration of $7.6 \mu\text{moles/ml.}$ Figure 6 shows results from a run of 890 hr at a culture at pH 6.4. When the dilution rate was set above 0.443 hr^{-1} , at $D 0.460 \text{ hr}^{-1}$ and 0.485 hr^{-1} , the culture turbidity decreased rapidly to 8.5 and 6.5 units, respectively; but at each dilution rate the rapid decrease was followed by a slow decline at the rate of about 1 unit in 15 hr, so a true steady state was not achieved. At the dilution rate 0.485 hr^{-1} the maltose remaining in the culture was $1.93 \mu\text{moles/ml.}$ At the other dilution rates the average amount remaining was $0.58 \mu\text{moles/ml.}$; this corresponds to a maltose utilization of about 92%.

Total counts of bacteria were made during continuous culture of *Bacteroides amylophilus* at different culture turbidities and dilution rates. A linear relationship was found between turbidity and total count; a turbidity of 8 corresponded to a total count of 3.4×10^9 bacteria/ml.

Figure 7 shows the values of Y (per mole of maltose utilized) obtained during the above culture and during another of 1100 hr duration. Figure 8 shows the volatile fatty acids formed at different dilution rates; these acids were identified as acetic and formic in equimolar amounts. The succinic acid, the only other fermentation product, formed was determined at two dilution rates. At $D 0.296 \text{ hr}^{-1}$ fermentation products, per $100 \mu\text{moles maltose used}$, were $172 \mu\text{moles formic acid}$, $172 \mu\text{moles acetic acid}$ and $159 \mu\text{moles succinic acid}$. Assuming uptake of CO_2 in succinic acid formation this gives a carbon recovery of 82.6%. At $D 0.077 \text{ hr}^{-1}$ the products were $126 \mu\text{moles formic acid}$, $126 \mu\text{moles acetic acid}$ and $201 \mu\text{moles succinic acid}$, giving a carbon recovery of 81.7%.

The Eh value of the medium as measured by the platinum and calomel electrode system was about -100 mV. at pH 6.5 (an actual reading of about -350 mV.), and the resazurin was entirely colourless. At this Eh value growth of the bacteria took place, but this growth did not result in any decrease of the Eh value, and when the Eh value increased, for any reason, the growth of the bacteria ceased and they began to wash out. The bacteria appeared to have no capacity for reducing the medium even when a heavy concentration of bacteria was present, although a (presumptive) *Bacillus* contaminant rapidly decreased the Eh value, even when present only in small numbers.

The morphology of the *Bacteroides amylophilus* bacteria in batch and continuous cultures varied from small cocci to small rods, with sometimes some of the irregularly shaped bacteria first noted by Hamlin & Hungate (1956). The number of irregular-shaped organisms increased under adverse conditions, such as high Eh value or low

pH value at the end of batch cultures; but the coccoid or rod-like morphology appeared to be independent of the growth rate. The same relationship between culture turbidity and bacterial dry weight was found whether the bacteria were predominantly coccoid or rod-shaped. The bacteria were capsulated at all times, but at dilution rates round about 0.3 hr^{-1} the culture foamed more than at lower or higher rates, and this seemed to be associated with an increase in capsule and slime production, although no marked difference in the nitrogen content (about 9–10% of the dry weight) could be found in bacteria grown at dilution rates of 0.148 , 0.222 and 0.354 hr^{-1} .

During two continuous cultures of *Bacteroides amylophilus* determinations were made of bacterial-nitrogen and total-nitrogen in culture supernatant fluid and in-flowing medium and ammonia-nitrogen in culture supernatant fluid and medium. Six

Fig. 7

Fig. 7. Culture of *Bacteroides amylophilus* on maltose. Results from two cultures of 890 hr and 1100 hr duration. Y_{maltose} is calculated on the basis of maltose used, not just that fermented (see text).

Fig. 8

Fig. 8. Culture of *Bacteroides amylophilus* on maltose. Volatile fatty acid (VFA) production. Results from the two cultures shown in Fig. 7.

determinations were made at dilution rates of 0.10 and 0.21 hr^{-1} . These showed that the ammonia disappearing from the medium was equivalent to the bacterial nitrogen formed ($\text{NH}_3\text{-N}$ was an average of 104% of cell N), so that the bacterium was using ammonia in preference to the amino acids of the tryptose. This type of nitrogen utilization has been found with other rumen bacteria (see review by Hungate, Bryant & Mah, 1964). Although the ammonia in the medium was rather less than that calculated from the constituent salt concentrations it was still present in excess in the culture and so could not have been limiting the growth of the bacteria.

The original strain of *Bacteroides amylophilus* used in the cultures described above changed in stock cultures in that it became slower growing, and it was found impossible to run continuous cultures over the range of dilution rates previously used. Further cultures were then made with a second strain which had been classified by the usual tests as identical to the previous one. This second strain grew over a similar range of dilution rates and produced the same fermentation products and enzymes, but it

differed in the slope of the culture turbidity/dry weight relationship, giving a higher turbidity for the same dry weight. Also at dilution rates round about 0.3 hr^{-1} the cultures foamed more than did the previous ones and at the high culture turbidities found here some 30% of the bacterial mass was a slime excreted into the culture medium. This second strain was used to continue and confirm the observations on enzyme activities begun with the previous strain. In a run of 1000 hr the total amylase and protease activities per ml. of the culture were measured at seven dilution rates between 0.09 and 0.41 hr^{-1} and corrected to activity per unit weight of bacteria in the culture. Both the protease and amylase activities showed a peak at a dilution rate about 0.2 hr^{-1} and the amylase activity showed a second peak at 0.09 hr^{-1} or less. A culture was run at a dilution rate of $0.283 \pm 0.006 \text{ hr}^{-1}$ for 490 hr and the culture was adjusted to seven values between pH 5.1 and 6.8. At pH 6.8 the culture washed out and at pH 5.1 a very granular growth occurred with a variable culture turbidity. At all intermediate pH values the culture turbidity was the same. Amylase activity showed a maximum at a culture pH of 6.1. Protease activity increased rapidly between pH 6.6 and 6.4 and then showed a slight increase as the culture changed to pH 5.1. The fermentation products also changed, the amount of volatile fatty acids produced per mole of maltose utilized showing a maximum at pH 5.8. Succinic acid was not determined.

DISCUSSION

The results given here again show that it is possible to grow strictly anaerobic bacteria for prolonged periods in continuous culture, but the results of the Eh measurements indicate why care must be taken to eliminate even the slightest traces of oxygen from both continuous and batch cultures. The inability of *Bacteroides amylophilus* to reduce the medium agrees with some unpublished experiments where we found that suspensions of *Selenomas ruminantium*, Bacterium 5S, *Ruminococcus albus* and *B. amylophilus* showed no oxygen uptake in manometric experiments, whereas rumen anaerobes such as *Veillonella* species and *Peptostreptococcus elsdenii*, which can grow at higher culture Eh values than the former bacteria, showed a rapid oxygen uptake, as did the clostridia which were tested. Presumably bacteria such as these latter, which are found in comparatively small numbers in the rumen, together with some facultative anaerobes, are responsible for the oxygen uptake of rumen contents and the preservation of an Eh suitable for the predominant bacteria (typified by the former group) in spite of the air introduced into the rumen during feeding. In cases where volatile fatty acids (VFA) and lactic or succinic acids are the fermentation products the proportions of VFA to lactic or succinic acids alter with growth rate. The results obtained here with pH-controlled cultures support the suggestion made previously (Hobson, 1965*a*) that the pH range for optimum growth of bacterium 5S is small (about pH 5.7–7). *B. amylophilus* will grow at a lower pH than 5S or *S. ruminantium* (Hobson, 1965*a*) and although the rumen pH value of the conventionally fed animal does not usually go below about pH 5.7 the rumen pH of animals fed on high-starch rations in which *B. amylophilus* may be important often decreases to as low as 5. Again confirmed are the comparatively low growth rates of the anaerobic rumen bacteria; and the maximum growth rates in batch cultures compared well with those obtained in continuous cultures. In the case of Bacterium 5S the concentration of carbon dioxide in the culture atmosphere had a profound effect on growth rate, but not on yield of bacteria or

fermentation products. Why this should be so is not apparent. Growth of *Bacterium* 5S was also slower on fructose than on glycerol.

Hobson & Summers (1966) reported the presence of two peaks for lipase and esterase production at different growth rates for *Bacterium* 5S growing on fructose. The present results show that this also held for growth on glycerol. Enzyme production was also a maximum in a culture at pH 6.6, which was about the optimum pH value for growth. The results obtained with *Bacteroides amylophilus* indicate that the amylase and protease activities of this bacterium also vary with growth rate, and with culture pH value. The evidence of changes in pattern of fermentation products with growth rate presented here and in the previous paper (Hobson, 1965*a*) is indicative of change in production of the enzymes of the fermentative pathways. Tempest & Herbert (1965) observed variations in rates of synthesis of the 'constitutive' oxidative enzyme systems of *Torula utilis* at different growth rates. Dawson (1965) observed that the intracellular amino acid pool of *Candida utilis* varied with growth rate during C- and N-limited chemostat culture, and Wright & Lockhart (1965) observed variations with growth rate in the antigenic composition of *Escherichia coli* organisms. There is thus increasing evidence for changes in the enzymic as well as the structural make-up of bacteria with growth rate as well as during growth between cell divisions.

The curve obtained by plotting bacterial concentration against dilution rate is of similar shape in all cases and like that found in previous experiments (Hobson, 1965*a*). However, in the case of *Bacterium* 5S growing on glycerol under 100% CO₂ there was a rather more abrupt washout at the higher dilution rates than found previously. This may have been due to a better control of pH value in the present apparatus. At dilution rates above 0.3 hr⁻¹, in the previous experiments the pH value rose from 6.2 to 6.6 and as this latter value is nearer the optimum for growth it may have given steady states at rather higher dilution rates than those found here. The decrease in bacterial concentration and bacterial yield at low dilution rates suggests a maintenance requirement for energy at low growth rates. Curves for bacterial concentration and dilution rate similar in shape to those found for the rumen bacteria have been found by other workers with different organisms and substrates (e.g. Wase & Hough, 1966); perhaps this type of relationship is more common than that found for *Aerobacter cloacae* by Herbert, Elsworth & Telling (1956), for which they derived theoretical equations.

In the previous paper (Hobson, 1965*a*) the bacterial yield (Y_{glucose}) of *Selenomonas ruminantium* was shown to be about 62 g./mole glucose fermented at optimum growth rates, and Hungate (1963) reported a value of about 55 g. bacteria/mole hexose fermented for *Ruminococcus albus* growing at one dilution rate on cellobiose with ammonia as nitrogen source. The results of the present work show that *Bacterium* 5S has a Y_{fructose} of 60 at optimum growth rate. *Selenomonas ruminantium* and *Bacterium* 5S need amino acids and were growing in a complex medium. *Bacteroides amylophilus* utilises ammonia as nitrogen source, and the fermentation balance suggests some 20% of the maltose was used as a source of bacterial carbon. The yield of *B. amylophilus* at optimum growth rate then becomes 160 g./mole maltose fermented (from Fig. 7). The fermentation pathways of these bacteria have not been investigated in detail, but assuming that 2 ATP are generated in the production of 2 pyruvate from hexose and that 1 ATP is formed in the conversion of pyruvate to acetate, propionate or succinate, we obtain values of Y_{ATP} of about 20 for *B. amylophilus* or *S. ruminantium*.

and 15 for *Bacterium* 5S growing on fructose. These values are higher than 10, which is the universal value suggested initially by Bauchop & Elsdén (1960) and since found for some other bacteria. On the other hand the value for Y_{glycerol} for *Bacterium* 5S (growing on glycerol) found here and in the previous experiments is 20, which corresponds to a value for Y_{ATP} of 10, assuming that 2 ATP are formed in glycerol fermentation. These calculations also assume that the enzymes liberated into the culture medium constitute a negligible proportion of the protein synthesized by the bacterium. This would appear to be the case with *B. amylophilus* since the bacterial-nitrogen formed and ammonia-nitrogen utilized were similar. These results suggest that Y_{ATP} may not be a constant even at optimum growth rates for different bacteria, and with individual types of bacteria it will obviously vary with growth rate, if some energy is used for maintenance and not bacterial growth. This latter variation again emphasizes the point that the bacterial yields in batch cultures, which we found to be less in these experiments than under optimum continuous culture conditions, cannot generally be taken as a guide to maximum yield when a maintenance requirement is involved, since they are a 'synthesis' of bacterial yields at different growth rates during the culture cycle, few if any of which may be near optimum. The high yields of *B. amylophilus* and *R. albus* (Hungate, 1963) suggest that little ATP energy is needed for the synthesis of cell constituents from simpler compounds, in conformity with the results of Senez (1962).

The provision of a grant from The Wellcome Trust, for part of the equipment, is gratefully acknowledged. We are also indebted to our colleague Dr T. H. Blackburn for the strains of *Bacteroides amylophilus* used here.

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