Growth of *Bacteroides fragilis* in Continuous Culture and in Batch Cultures at Controlled pH

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Bacteroides fragilis NCTC 9343 has been grown in continuous cultures with glucose as growth-limiting factor. At pH 7.0 and at a dilution rate of 0.07 per h, glucose limited growth in concentrations up to 0.6%. Maximal cell yield and productivity were obtained with 0.87% glucose in the inflowing medium. A pH of 7.0 was optimal for growth. With 0.6% glucose in the fresh medium and at pH 7.0, cell yield and productivity were highest at a dilution rate of 0.07 per h and 0.11 per h, respectively. At dilution rates higher than 0.07 per h, glucose was no longer growth limiting, and at dilution rates above 0.11 per h, another compound seemed to have replaced glucose also as energy source. When grown in batch cultures at pH 7.0, the best yields of *B. fragilis* was achieved with 0.6% glucose in the fresh medium. The highest specific growth rate (μm) determined from viable counts was 0.45, corresponding to a mean generation time of 92 min.

The cell yield in cultures of *Bacteroides fragilis* can be increased by use of stirred fermentors (13). It has also been shown that anaerobic gram-negative bacteria can be grown under carbohydrate-limiting conditions in continuous cultures for long periods (6, 7, 12). In the case of *Selenomonas ruminantum*, the yield was considerably higher in continuous culture at optimal growth rate than in batch culture (6).

Continuous cultivation of *B. fragilis* was initiated for two purposes. The first was to elaborate a technique for cultivation of bacteria in high yields, which also was time saving. The second was to obtain bacteria for immunochemical studies which had been grown under controlled conditions in a chemical and physical environment invariable with time.

The present report is concerned with growth of *B. fragilis* NCTC 9343 in the chemostat at varying pH and dilution rates, and at different concentrations of the growth-limiting factor which was glucose. Results of preliminary batch cultivations at controlled pH are included.

MATERIALS AND METHODS

Organism. The microorganism used was *B. fragilis* NCTC 9343.

Medium. The basal medium contained whale meat extract (Rieber and Sön A/S, Bergen, Norway), 10 g; proteose peptone (Oxoid), 15 g; NaCl, 5 g; KH_2PO_4, 1.5 g; $Na_2HPO_4 \cdot 2H_2O$, 3.5 g; $(NH_4)_2SO_4$, 0.5 g; and L-cysteine hydrochloride, 1 g/liter. Solutions of glucose were sterilized separately (120 C, 30 min) and added aseptically to the basal medium which had been sterilized at 120 C for 60 min on 2 consecutive days.

Continuous cultivation. A stirred fermentor with a working volume of 3 liters (BioTec FT 104, BioTec AB, Stockholm, Sweden) and the Instrumentation Panel type LP 100 was used. The panel comprised the following units: a stepless drive unit for regulation of stirrer rate, units for automatic maintenance of constant pH (pH-meter, type PHM 41, Radiometer AS, Copenhagen) and temperature in the culture vessel, a peristaltic pump unit for feeding of medium, and units for control of gas flow into the vessel and of foam level. The set up was in essentials as described by Wahren, Bernholm, and Holme (12). The accuracy of the pump rate was ±4%. NaOH (2 N) was used for neutralization. The pH electrode (Radiometer GK 2302 C) was sterilized by immersion for 4 h in a solution of 175 ml of ethanol, 1.75 ml of H_2SO_4, and 75 ml of distilled water. The fermentor with 1 liter of medium in the culture vessel and rubber and glass tubings for connecting the fermentor to the 5-liter medium reservoir, to the NaOH solution, and to a sampling flask were sterilized at 120 C for 60 min and assembled aseptically. The fermentor was filled up with medium and checked for sterility, and the bubbling of gas (7% CO_2, 93% N) through the culture vessel was started 4 h before the vessel was inoculated with bacteria and the control units were put into function. The pH of the culture was checked twice daily by independent measurements on a Metrohm type E 300 pH-meter (Metrohm AG, Herisau, Switzerland). The temperature (37 ± 0.1 C), the stirrer rate (300 rpm), and the gas flow (2 liter per h) were the same in all experiments. Addition of antifoam agent was not necessary.

Bacterial dry weight. Measurements were made in quadruplicate and averaged. Samples (25 ml) of culture were centrifuged for 20 min at 8,000 × *g*, washed once in 0.01 M phosphate buffer, pH 7.0, and freeze-dried in preweighed tubes.

Optical density. Density measurements were made at 600 nm using a Spectrochem MK 2 spectrophotometer (Hilger and Watts Ltd., London, England). All readings were made against sterile medium. The relationship between optical density and bacterial dry weight was linear up to 1 mg (dry weight) per ml of culture. The ratio of optical density (600 nm) per milligram (dry weight) over the linear range was 1.3.

Bacterial cell count. The number of viable cells was counted in culture samples diluted 10-fold in brain heart infusion supplemented with 0.1% L-cysteine hydrochloride. In quadruplicate, 0.01 ml of each dilution was spread with a standard volume-calibrated loop (4) on a series of freshly prepared blood agar plates. Incubation was performed for 2 days at 37 C in an atmosphere of 7% CO_2, 10% H_2, and 83% N_2.

Growth parameters. The yield coefficient Y_{glu} was calculated according to Bauchop and Elsden (1): Y_{glu} = grams (dry weight) of bacteria produced/mole of glucose utilized.

The utilization of glucose in a balanced continuous culture is given by the difference in glucose concentration in the medium reservoir and the effluent. The growth in the basal medium, without glucose, was barely measurable by optical density readings and has been neglected in calculating Y_{glu}. The dilution rate *D* is the liquid flow rate divided by the total medium volume in the fermentor and has the unit per hour. The product *D* × (dry weight) bacteria gives the productivity, i.e., the cell mass produced in a given culture volume during 1 h.

The specific growth rate in batch cultures was calculated from the formula:

$$\mu m = 2.3 \times ([\log P_t - \log P_{to}]/t - t_o)$$

where $t - t_o$ is the time interval during which the bacterial cell population P_{to} increased to the value P_t. The population of cells was determined from bacterial cell counts. The mean generation time *g* was calculated as: $g = \ln 2/\mu m$.

Glucose assays. Quantitative determination of glucose in the medium and in the cultures was performed by use of glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, N.J.).

RESULTS

Batch cultures at controlled pH. Batch cultivation of *B. fragilis* NCTC 9343 was performed at pH 7.0 with constant stirrer rate and gas flow, and with 0.05, 0.24, 0.50, 0.54, 0.58, 0.78, or 1.35% glucose in the medium. In all experiments, the inoculum was 40 ml of an overnight culture of NCTC 9343, grown at 37 C in fluid thioglycolate medium (Difco), and

stored at 4 C for 1 day (while sterility was checked). This inoculum gave a concentration of about 5 × 10⁶ microbial cells per ml of growth medium. The cultures were left undisturbed for 8 to 10 h. Thereafter, small samples were drawn every 2 to 3 h for counting of viable cells and ·measurements of optical density. As far as possible, storage of the samples, which were kept in an ice bath, was avoided. Parts of the samples were centrifuged and the supernatant was used for determination of glucose. The determinations were done immediately or in supernatants stored at −25 C. The samples were also examined for contaminating microorganisms by Gram staining and aerobic cultivation on blood agar.

The cultures started growing 8 to 10 h following inoculation and grew exponentially for about the same period of time. Then the growth slowed down and ceased when all glucose became exhausted in the medium. With all cultures this took place within 30 h. As soon as the stationary phase of growth was reached, the number of viable cells began decreasing. A typical experiment is shown in Fig. 1.

The cell yield, calculated as dry weight from optical density readings, increased linearly with concentrations of glucose up to 0.58% (Fig. 2). In contrast, the yield coefficient Y_{glu} decreased with increasing amounts of glucose in the medium.

The specific growth rate of exponentially growing cultures was determined from viable counts (Table 1). The highest values were obtained with 0.54% glucose in the medium,
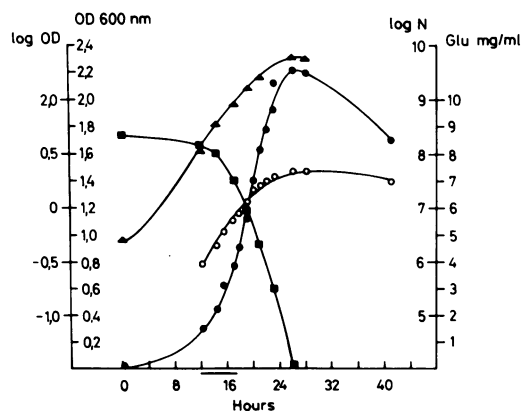

FIG. 1. *Growth of NCTC 9343 in a stirred fermentor at pH 7.0. Glucose (0.87%) was in the fresh medium. Symbols:* ●, *optical density;* ○, *log optical density;* ▲, *log viable count (N);* ■, *concentration of glucose in the culture. The horizontal line just below the abscissa denotes the time interval at which the specific growth rate was determined (see Table 1).*
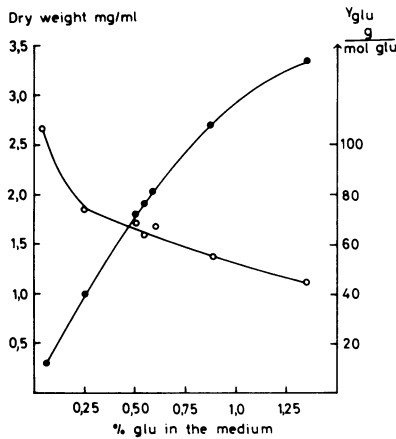
FIG. 2. Cell yield (●) and yield coefficient Y_{glu} (O) in cultures of NCTC 9343 grown at pH 7.0 with various concentrations of glucose in the fresh medium.

TABLE 1. Specific growth rate (μm) and mean generation time of NCTC 9343 grown in a stirred fermentor at pH 7.0

Glucose in the medium (%)	Specific growth rate (μm)	Generation time (min)
0.05	0.12	345
0.24	0.34	122
0.50	0.43	96
0.54	0.45	92
0.58	0.42	98
0.87	0.32	129
1.35	0.30	138

and corresponds to a mean generation time of 92 min.

Continuous cultures. NCTC 9343 was cultured in the chemostat at constant stirrer rate and gas flow and with glucose as growth-limiting factor. The growth vessel was inoculated with 100 ml of an 18- to 20-h culture grown at 37 C in fluid thioglycolate medium (Difco) and stored overnight at 4 C. The cultures were grown batch-wise until the following day when the continuous flow was started. When changing from one growth condition to another, the cultures were left to equilibrate until 15 liters of fresh medium had flowed through the vessel and the optical density remained constant in samples taken at intervals. A large sample (2 to 3 liters) was then collected from the overflow tube into a receiver surrounded by ice, and parts of the sample were used for determination of dry weight and glucose. The cultures were checked daily for contaminating bacteria by Gram staining and cultivation on blood agar.

Cell yield and productivity were studied in cultures grown at a dilution rate of 0.07 per h, at pH 7.0, and with 0.20, 0.38, 0.55, 0.63, 0.69, 0.87, 1.05, or 1.4% glucose in the inflowing medium. The yield of bacteria as estimated by dry weight and optical density, and the productivity increased proportionally with glucose concentrations up to 0.63% (Fig. 3). The highest values were obtained with 0.87% glucose in the fresh medium. When the concentration of glucose was increased to 1.05%, the cell yield decreased slightly and all the added glucose was not utilized by the growing bacteria. The yield coefficient Y_{glu} decreased with rising concentrations of glucose.

The influence of pH on cell yield and productivity was studied in cultures grown at a dilution rate of 0.07 per h (Fig. 4). Optimal pH was 7.0. Cell yield and productivity decreased markedly when pH was raised to 7.5.

Continuous cultures were also studied at varying dilution rates (Fig. 5). The highest yield (1.57 mg/ml) was obtained at a dilution rate of 0.07 per h. A dilution rate of 0.11 per h was optimal for productivity. At dilution rates higher than 0.16 per h, the cultures washed out. The yield coefficient rose slowly with increasing dilution rate up to 0.11 per h, and thereafter abruptly.
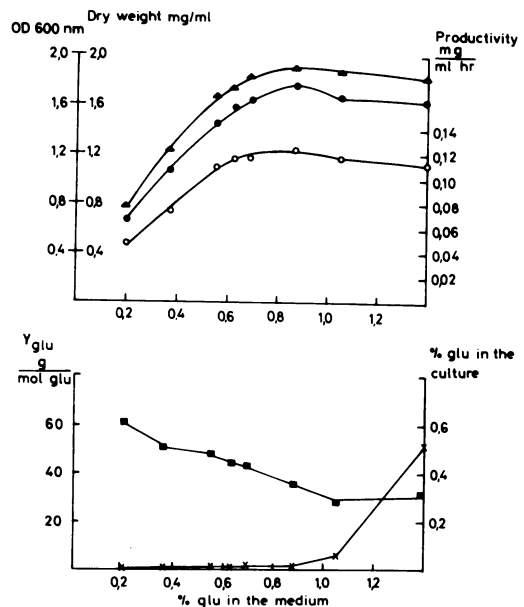

FIG. 3. Growth of NCTC 9343 in continuous cultures with various amounts of glucose in inflowing medium. pH, 7.0; D, 0.07 per h. Symbols: ●, bacterial dry weight; ▲, optical density; O, productivity; ■, yield coefficient; ×, concentration of glucose in the culture.
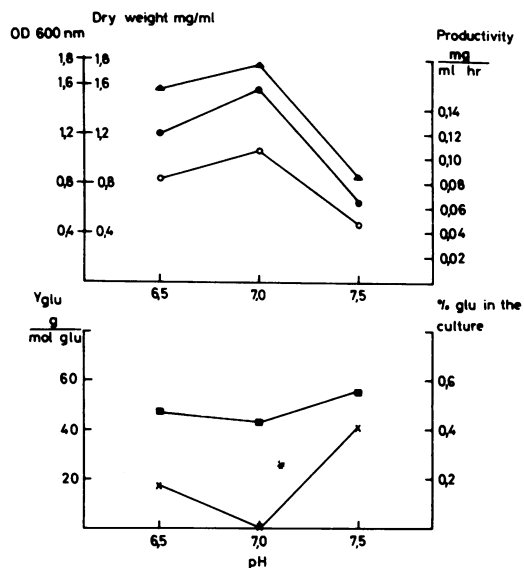
FIG. 4. *Growth of NCTC 9343 in continuous cultures at different pH values. D, 0.07 per h. Percentage of glucose in inflowing medium was 0.61 (pH 6.5), 0.65 (pH 7.0), and 0.62 (pH 7.5). Symbols: see legend to Fig. 3.*

No difficulties were experienced with "wall growth" in continuous cultures run for up till 4 weeks. The reproducibility of the experiments was good.

Microbial morphology changed with increasing influent glucose concentrations and with raising dilution rates up to 0.13 per h from small coccoid, detached cells to chains of rods about 6 μm long.

DISCUSSION

The yield obtained in this laboratory by batch cultivation of *B. fragilis* NCTC 9343 without stirring and control of pH·has varied between 0.8 and 1.0 mg of dry cells per ml of medium. By using a stirred fermentor and pH control, the yield was more than doubled. Similar findings have previously been reported by Wahren et al. (12). The growth was proportional to glucose added between 0.05 and 0.58%. The figures for specific growth rate (Table 1) indicates a saturation constant K_s for glucose which is considerably higher than that found by cultivation of aerobic bacteria. Optimal growth rates were observed with 0.50 to 0.58% glucose in the fresh medium. Higher glucose concentrations may have a slight growth inhibitory effect. This assumption is supported by results obtained previously (unpublished data) by batch cultivation of NCTC 9343 without pH control.

By such cultivation the yield of bacteria decreased when glucose was added to the medium in concentrations higher than 0.75%.

The present study shows that it is possible to grow *B. fragilis* NCTC 9343 for prolonged periods in continuous culture using the energy source as growth limiting factor. Under the experimental conditions used, glucose limited growth in concentrations up to 0.6%, as evidenced by a linear increase in cell yield and culture density. Decreasing yield coefficient with rising concentrations of growth-limiting factor in inflowing medium has also been observed by cultivation of *Streptococcus faecalis* under anaerobic conditions (9). This may be due to higher consumption of energy for maintenance with increasing culture density (8). Optimal growth of NCTC 9343 in continuous culture at pH 7.0 is in accordance with results obtained by batch cultivation (12).

The curves obtained by plotting optical density and cell yield against dilution rates had a shape similar to those found by continuous cultivation of *B. amylophilus* and other anaerobic rumen bacteria (7). This type of relationship differs from theoretical growth in continuous cultures. According to Herberth et al. (5), both cell yield and the yield coefficient should remain constant irrespective of the dilution rate
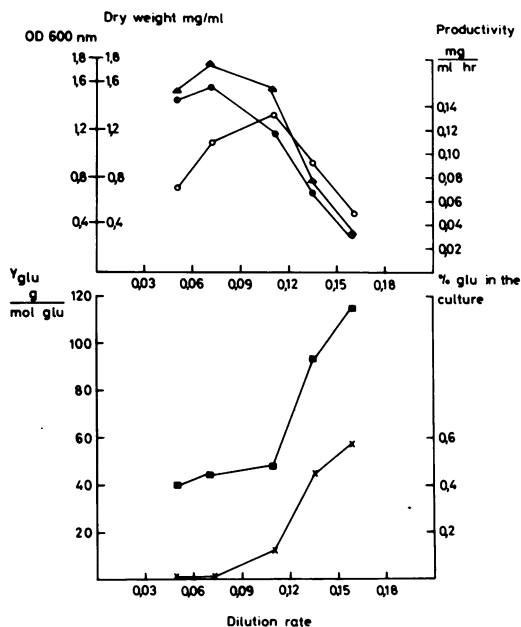

FIG. 5. *Growth of NCTC 9343 in continuous cultures at different dilution rates. pH, 7.0. Percentage of glucose in inflowing medium was 0.60 (D, 0.05), 0.63 (D, 0.07), 0.56 (D, 0.11), 0.58 (D, 0.14), and 0.62 (D, 0.16). Symbols: see legend to Fig. 3.*

as long as washout does not occur. An increase of the yield coefficient with a rise in growth rates has been frequently observed (6, 9–11). When the energy source is growth limiting, this is thought to be due to a relative decrease of the amount of energy utilized for maintenance of the cultures at high growth rates (8).

At dilution rates above 0.07 per h, glucose was possibly no longer a growth-limiting factor. All added glucose was not utilized, the cell yield decreased, and wash-out appeared at a relatively low dilution rate. The yield coefficient, however, increased considerably with dilution rates exceeding 0.11 per h. At a dilution rate of 0.16 per h, the amount of glucose utilized per gram of bacteria produced was as little as 0.009 mol, indicating that another compound had replaced glucose also as energy source. This suggests that in the present experiments the dilution rate influenced not only the rate of growth, but had a qualitative effect on bacterial metabolism. A qualitative change in glucose metabolism with growth rate has been observed in continuous cultures of *Aspergillus nidulans* (2).

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