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Growth of *Bacteroidaceae* in Stirred Fermentors

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The conditions for increasing bacterial yields in cultures of *Bacteroidaceae* by the use of stirred fermentors and *p*H control were investigated by means of three representative species: *Sphaerophorus necrophorus*, *Bacteroides fragilis*, and *B. melaninogenicus*. A medium containing tryptone, yeast extract, and glucose or sucrose was used. Horse serum had to be added to obtain substantial growth of *B. melaninogenicus*. The optimal *p*H for growth rate and yield was 7.0 to 7.2. Lysis of the bacteria occurred when the glucose (*or sucrose*) was exhausted. The rate of lysis was very high in cultures of *S. necrophorus*, less so in *B. fragilis* and *B. melaninogenicus*. Pleomorphism, manifested as large spherical forms of the bacteria, was observed in the late logarithmic phase of *S. necrophorus*. Great differences in the length of the lag phase and of the mean generation time were found among the three bacterial species. The yield in static cultures of the three species without *p*H control was approximately 0.4 g of dry cells per liter, but was increased, in stirred fermentors with *p*H control, to 3.5 g (*S. necrophorus*), 2.7 g (*B. fragilis*), and 4.3 g (*B. melaninogenicus*) per liter. With an inoculum density of 5 to 10 mg (dry weight) per liter, these yields were obtained in approximately 10 (*S. necrophorus*), 25 (*B. fragilis*), and 35 hr (*B. melaninogenicus*), respectively.

Various *Bacteroidaceae* species are present in large amounts in the normal oral and intestinal flora of man (3, 8, 18, 20). These bacteria have also been shown to be associated with various infections in man (1, 3, 5, 14, 19). Despite their abundance in the normal flora of man, comparatively little is known about their metabolism and physiology. One of the reasons for this scarcity of knowledge is that most *Bacteroidaceae*, being fastidious organisms, are difficult to grow in high yields and in pure cultures. This is especially true for those members of this group which occur in the normal flora of the human bowel. In most of the earlier studies, these bacteria were grown in static cultures which usually had to be incubated for several days to obtain visible growth. The purpose of the present study was to find a simple and preferably rapid method of cultivating selected *Bacteroidaceae* in high yields to produce material for studies of their enzymes, and to grow these bacteria under well-defined conditions in order to create a suitable system for metabolic studies.

MATERIALS AND METHODS

Bacterial strains. *Bacteroides fragilis* 9343, *B. necrophorus* 7155 (*Sphaerophorus necrophorus*), and *B. melaninogenicus* 9338 were obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London, England.

Media. The basal medium used contained (per liter): yeast extract (Difco), 5 g; tryptone (Oxoid), 15 g; sodium thioglycolate, 0.5 g; sodium chloride, 2.5 g; L-cystine, 0.5 g; and methylene blue, 0.02 g. When required, hemin was added at 5 μg/ml and menadione at 0.25 μg/ml. Generally, glucose was used as the energy source in concentrations between 5 and 15 g/liter. All media were freshly prepared and sterilized immediately before use. A gas mixture (95% nitrogen and 5% carbon dioxide) was bubbled through the medium to assure an anaerobic environment.

Cultivation. Batch cultures were grown at 37 C in 4-liter stirred fermentors (Biotec FL 103, Biotec AB, Stockholm, Sweden) and similar fermentors of 1-liter capacity equipped with temperature and *p*H control (Radiometer titrator TTT1). The cultures were titrated with 2 N sodium hydroxide. An anaerobic atmosphere was maintained by bubbling a gas mixture of 95% nitrogen and 5% carbon dioxide through the culture at a rate of 2 liters/hr. Samples (15 ml) were taken from the cultures during growth for the determination of cell dry weight, glucose concentration in the medium, and volatile acidic end products. Dry weights were performed on 10-ml samples. The organisms were harvested by centrifugation, washed once in 0.01 M phosphate buffer (*p*H 7.0), and dried at 100 C prior to weighing. All weights were corrected for the weight of the buffer salts.

S. necrophorus and *B. fragilis* cultures were inoculated from fresh fluid cultures grown in test tubes to give an initial cell concentration of approximately 10 mg/liter. *B. melaninogenicus* cultures were initially inoculated from blood-agar slants. In later experi-

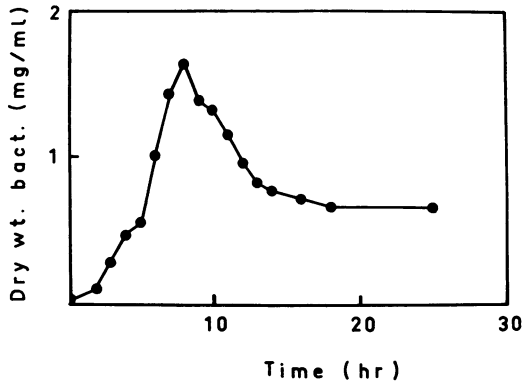

FIG. 1. *Growth of B. necrophorus in the basal medium with a glucose concentration of 10 g/liter.*

ments the cultures were inoculated from fresh fluid cultures. Both methods were adapted to give an initial cell density of approximately 10 mg/liter in the final cultures.

Chemical analyses. Glucose was analyzed enzymatically (AB Kabi, Stockholm, Sweden) by use of a Technicon Auto-Analyzer. The spent medium was analyzed for volatile fatty acids after protein precipitation by $Zn(OH)_2$ according to Neish (16). A Perkin-Elmer model F 6 gas chromatograph was used for determination of the volatile fatty acids. The column filling was Porapak Q plus 3% H_3PO_4. The column temperature was 212 C, and the carrier gas (N_2) flow rate was 60 ml/min.

RESULTS

Three species of *Bacteroidaceae*, *Sphaerophorus necrophorus*, *B. fragilis*, and *B. melaninogenicus*, were chosen for the present study as being the most prevalent in this group. Basically, the same cultivation equipment could be used to obtain good growth of all three species. The maintenance of a constant *p*H during cultivation was essential for high bacterial yields. The use of a stirred fermentor insured good mixing, which was of great importance, especially during the later part of the growth period, when large amounts of alkali were pumped into the vessel. The stirrer rate was usually 600 rev/min, but, since this was found to inhibit *B. melaninogenicus*, cultures of this organism were stirred at 100 rev/min. Since great differences in morphology, nutritional requirements, and metabolic characteristics were observed among the three bacterial species, the results are presented separately for each species.

S. necrophorus was first grown in the basal medium with the addition of 10 g of glucose per liter. A typical growth curve is shown in Fig. 1. The mean generation time during the log phase was 60 to 80 min. When the glucose concentration was increased to 15 g/liter, the maximal yields were obtained after 10 to 12 hr and amounted to 3.0 to 3.5 g/liter in three experiments. Growth ceased when the medium glucose was exhausted, and rapid lysis occurred thereafter. The maximal yields from sucrose (10 g/liter) were 3.4 and 3.6 g (dry weight) per liter for the two cultures after approximately 10 hr, followed by lysis. Serum (20 ml/liter), as well as hemin and menadione, were added to the medium singly and in combination. None of these substances influenced growth rate or cell yield and they were therefore excluded from the medium.

Optimal *p*H for growth was 7.0. Considerably lower yields were obtained at *p*H 6.5 and 7.5. When not controlled, *p*H decreased to 5.7 in the cultures with 10 g of glucose per liter. An average of 65 ml (initial glucose concentration, 10 g/liter), 115 ml (15 g of glucose), or 100 ml (10 g of sucrose) of 2 N sodium hydroxide per liter, respectively, was used to maintain a constant *p*H. Acetic and *n*-butyric acids were the main acidic end products. *S. necrophorus* underwent a marked morphological change during growth (Fig. 2).

B. fragilis was first grown in the basal medium containing 10 g of glucose per liter. A typical growth curve is shown in Fig. 3. The mean generation time during log phase was approximately 300 min. When the glucose concentration

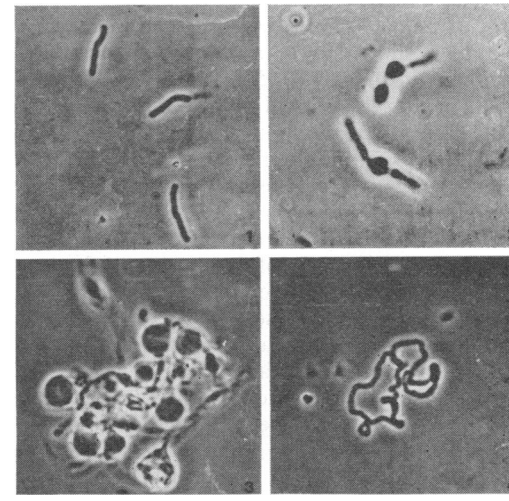

FIG. 2. *Morphology of B. necrophorus at different phases o· growth.* (1) *Single, rod-shaped cells in the early logarithmic phase;* (2) *appearance of spherical bodies in elongated cells in the middle of the logarithmic phase;* (3) *clustered elongated cells and filaments with spherical bodies at the end of the logarithmic phase;* (4) *irregular chains at 4 hr after the start of lysis. No spherical forms are seen. Phase-contrast microscopy.* ×*4,000.*

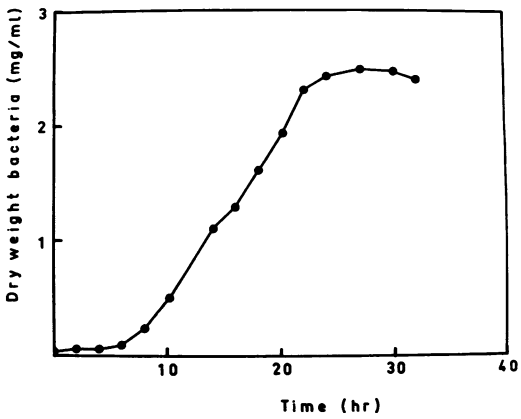

FIG. 3. *Growth of B. fragilis in the basal medium with a glucose concentration of 10 g/liter.*

was increased to 15 g/liter, a maximal cell yield of 2.5 g/liter was achieved in 23 hr. Growth ceased when glucose was exhausted. When sucrose (10 g/liter) was used as the energy source, the length of the lag phase was increased to 12 to 14 hr. A maximal cell yield of 2.7 g/liter was obtained after 26 hr. The growth rate was accelerated in this case, the generation time being approximately 100 min. Addition of hemin and menadione to the medium appeared to induce a slightly faster growth rate but did not increase the final cell yield. Neither cell yield nor growth rate was increased when serum (20 ml/liter) was added to the basal medium. Optimal *p*H for growth was 7.0. When not controlled, it fell to *p*H 5.0.

The average amounts of 2 N sodium hydroxide used to maintain a constant *p*H were 75 ml (initial glucose concentration, 10 g/liter), 95 ml (15 g of glucose, and 70 ml (10 g of sucrose) per liter. The main acidic end products formed were acetic, propionic, and isovaleric acid. No marked differences in cell morphology were observed during the different growth phases of this organism.

B. melaninogenicus grew poorly in the basal medium with 5 g of glucose/liter added; the maximal cell yield was 0.4 g/liter. Higher glucose concentrations did not improve the yield. The basal medium supplemented with filter-sterilized horse serum (5 to 50 ml/liter) was tested. The optimal serum concentration was found to be 40 ml/liter. Hemin and menadione were required for growth (11). A typical growth curve is shown in Fig. 4. The mean generation time in the logarithmic phase was approximately 200 min. In these experiments, glucose was not exhausted at the end of the logarithmic phase and its concentration exceeded 1 g/liter in all experiments. When the serum concentration was 20 ml/liter, cell yields of 3.0 to 3.8 g/liter were obtained in 40 hr (mean generation time 300 min) and glucose was exhausted in the medium at the end of the logarithmic phase. Higher glucose concentrations did not increase the cell yield.

Optimal *p*H for growth was 7.0; *p*H 6.5 and 7.7 gave considerably lower cell yields. When not controlled, *p*H decreased to 6.2.

The amounts of 2 N sodium hydroxide used to maintain a constant *p*H were 35 to 45 ml (initial glucose concentration, 5 g/liter) and 55 to 65 ml (10 g of glucose) per liter. The main acidic end products formed were acetic, isobutyric, and isovaleric acid. No marked differences in cell morphology were observed during the different growth phases in cultures of this organism.

DISCUSSION

Yields in anaerobic cultures of bacteria are generally low compared to those obtained for aerobes, although there is considerable variation. The advantage of using a stirred fermentor for cultivation of anaerobes has been pointed out by Sargeant (17). Adequate mixing facilitates temperature and *p*H control. In addition, cultures in a stirred fermentor are homogeneous and are more reproducible than under static conditions. It also enables the correlation of cell morphology or production of enzymes to specific phases of growth.

The yields reported for cultures of *Bacteroides* species do not exceed 0.5 g (dry weight) per liter. This figure was reported by Blackburn (2), whereas a maximum yield of 5×10^8 bacteria per ml of culture was obtained by Gesner and Jenkin (10) corresponding to approximately 0.1

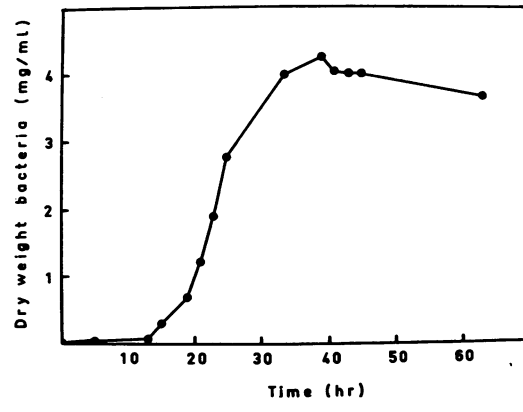

FIG. 4. *Growth of B. melaninogenicus in the basal medium supplemented with horse serum, glucose (5 g/liter), hemin, and menadione.*

TABLE 1. *Bacterial yields at the end of the logarithmic phase*[a]

Strain	Glucose (g/liter)					Sucrose (10 g/liter)	
	Serum		No serum			Serum	No serum
	5	10	5	10	15		
B. necrophorus		1.9		1.8	3.2		3.5
B. fragilis		2.4		2.5	2.5		2.7
B. melaninogenicus	4.3	4.1	0.4	0.6		No growth	

[a] Results expressed as grams per liter (dry weight). Horse serum was added to the cultures in a concentration of 40 ml/liter of medium.

g/liter. Cultivation of *Bacteroides* species for production of their enzymes and for immunological studies has been performed in static cultures without *p*H control (4, 10, 12, 13, 15). In the present study, similar conditions were used for the precultures, and the yields obtained for the three species were 0.4 g/liter. The time required to reach the stationary phase differed, however; after incubation for 18 hr, *S. necrophorus* gave the same density as did *B. fragilis* after 48 hr and *B. melaninogenicus* after 72 hr.

The yields obtained in the stirred cultures are summarized in Table 1. The bacterial concentrations were followed in some of the cultures by counting under the phase contrast microscope. The maximum yields reported in Table 1 correspond to 1.6×10^{10} bacteria per ml for *S. necrophorus*, 1.3×10^{10} for *B. fragilis*, and 2.1×10^{10} for *B. melaninogenicus*. From these figures it is possible to calculate the average dry weight per bacterium, which was the same for the three species: 2×10^{-10} mg in the stationary phase, approximately 5×10^{-10} mg in the early logarithmic phase.

The cause of the low yields generally obtained in anaerobic cultures is in most instances the development of a low *p*H, assuming that the growth medium contains all nutrients required in adequate concentrations. The use of *p*H control permits a substantial increase in yields. For anaerobic growth, however, large amounts of the energy source are required for cell synthesis, usually resulting in the production of organic acids. A large addition of alkali is required to maintain a constant *p*H, and it is possible that the buildup of a high concentration of inorganic ions retards the growth rate. Variation in tolerance of inorganic ions and organic acids between bacterial species would then result in yield differencies.

The glucose requirement varied among the species. For the synthesis of 1 g of bacteria (dry weight), *S. necrophorus* consumed approximately 5 g of glucose, *B. fragilis* consumed 4 g of glucose, and *B. melaninogenicus* less than 1 g. In *B. melaninogenicus*, comparatively small amounts of glucose were required for cell synthesis. However, the glucose concentration in the culture fluid decreased slowly throughout the period of active growth, indicating that glucose and other energy sources were used simultaneously. The alkali requirement per mole of glucose consumed was fairly constant for all cultures. The amount of sodium hydroxide added corresponded to approximately 2.5 moles of acid produced per mole of glucose. The fact that similar amounts of acids were produced per mole of glucose consumed, irrespective of the cell yields, indicates that glucose was used mainly as an energy source. In static cultures of all three species in media without glucose, a *p*H decrease of less than half a unit was observed. Thus, the influence of the use of energy sources other than glucose, such as amino acids, on the consumption of alkali appears to be of little significance.

The variations in yields recorded under the same conditions from different cultivations of the same bacterial species could partly be due to the lysis occurring after exhaustion of the glucose. If no sample was taken at the moment when lysis started, the yield figure obtained for that experiment would be too low. This error would be especially pronounced for *B. necrophorus*, which showed a high initial rate of lysis after exhaustion of the glucose.

The sources of nitrogenous constituents of the medium were calculated to be in excess, and the reason for the limitation of the yields of the different species remains to be investigated. The hemin and menadione requirement of *B. melaninogenicus* (11) indicates that very specific medium components may be needed by *Bacteroidaceae* organisms. In addition, a requirement for one or a few amino acids might be exceptionally high, if these are used as energy sources.

Serum was added to the medium to determine

whether it could protect against possible toxic effects of oxygen (6, 9). The results correlate well with the requirement for hemin, displayed only by *B. melaninogenicus*, since it has been suggested that hemin has a neutralizing effect on oxygen toxicity (7). The tolerance for molecular oxygen has been shown to differ widely among different anaerobic bacteria (9). Auto-oxidation of reducing agents, e.g., thioglycolic acid, in the presence of oxygen has been shown to lead to the formation of hydrogen peroxide, which might strongly inhibit the growth of these bacteria which do not produce catalase (7). The possibility that the serum protein can nonspecifically bind and neutralize other agents, e.g., toxic products of metabolism, must also be considered.

The use of stirred fermentors and *p*H control considerably improved the size and reproducibility of the bacterial yields obtained in cultures of *Bacteroides*. The results indicate, however, that further studies on the metabolism of these bacteria may lead to still better methods for their production.

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