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Formation of Glycosidases in Batch and Continuous Culture of *Bacteroides fragilis*

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Nine strains of *Bacteroides fragilis* were cultivated in stirred fermentors and tested for their ability to produce glycosidases. *B. fragilis* subsp. *vulgatus* B70 was used for optimizing the production of glycosidases. The highest bacterial yield was obtained in proteose peptone-yeast extract medium. The optimum pH for maximal bacterial yield was 7.0, and the optimum temperature for growth was 37°C. The formation of glycosidases was optimal between pH 6.5 and 7.5, and the optimum temperature for synthesis of glycosidases was between 33 and 37°C. Culture under controlled conditions in fermentors gave more reproducible production of glycosidases than static cultures in bottles. The strain was also grown in continuous culture at a dilution rate of 0.1 liter/h at pH 7.0 and 37°C with a yield of 2.0 mg of dry weight per ml in the complex medium. The formation of glycosidases remained constant during the entire continuous process.

Large numbers of several *Bacteroides* species are present in the normal human oral and intestinal flora (13, 15). Some of these species have been shown to cause various infections in humans (5, 7), but because most *Bacteroides* species are difficult to cultivate in vitro, little is known about their mechanism of virulence. Possible factors include cell envelope antigens, toxins, and various enzymes.

Different bacterial species in the normal flora in various parts of the body are capable of producing glycosidases. Under certain conditions, these enzymes split off various sugars from the glycoprotein molecules that make up the mucins (4). The sugars released could supply energy for the growth of certain bacteria, and therefore these enzymes might be important in the maintenance of the normal flora on mucosal surfaces.

The aim of the present study was to evaluate different prereduced media for growth of *Bacteroides fragilis* under conditions of defined pH, temperature, and agitation and to compare the formation of glycosidases under different growth conditions. Experiments to determine the optimum pH and temperature for growth and production of glycosidases were also carried out.

MATERIALS AND METHODS

Bacterial strains. *B. fragilis* subsp. *vulgatus* ATCC 8482, *B. fragilis* subsp. *ovatus* ATCC 8483, and *B. fragilis* subsp. *distasonis* ATCC 8503 were obtained from the American Type Culture Collection, Rockville, Md. *B. fragilis* subsp. *fragilis* NCTC 8560 and *B. fragilis* subsp. *thetaiotaomicron* NCTC 10582 came from the National Collection of Type Cultures, London, England. *B. fragilis* subsp. *ovatus* B4, *B. fragilis* subsp. *vulgatus* B70 and B72, and *B. fragilis* subsp. *fragilis* B153 were all isolated from patients with septicemia and identified according to Holdeman and Moore (9).

The anaerobic culture system (Bellco Glass, Inc., Vineland, N.J.) developed by the Anaerobe Laboratory, Virginia Polytechnic Institute and State University (9), was used for subculturing the strains. Reidentification of *B. fragilis* was made after every batch and continuous culture according to the method of Holdeman and Moore (9).

Preparation of prereduced media. Unless otherwise stated, the following basal medium was used: proteose peptone (Difco), 20 g; yeast extract (Difco), 5 g; cysteine-hydrochloride, 0.55 g; NaCl, 5 g; glucose, 10 g; resazurin solution, 6 ml (Merck, Darmstadt, Germany); and distilled water to a final volume of 1 liter. The pH of the medium was 7.0. All ingredients except cysteine-hydrochloride were mixed in a 7-liter Erlenmeyer flask. The medium was boiled for 20 min until the color changed from pink to yellow. The flask was then cooled in an ice-water bath under oxygen-free nitrogen, and cysteine-hydrochloride was added. The medium (1 liter) was dispensed under an anaerobic atmosphere into the fermentor, which was autoclaved for 20 min at 121°C.

Four other complex prereduced media (all containing 10 g of glucose per liter) were also tested: a peptone-yeast extract medium (9), a tryptone-yeast extract medium (21), a tryptone medium (22), and a whale meat medium (1). A prereduced defined medium described by Wahren and Holme (22) containing resazurin solution (4 ml/liter of medium) was also used.

Manipulations of all the media were performed in an anaerobic chamber (Coy, Ann Arbor, Mich.) under

a 95% (vol/vol) nitrogen and 5% (vol/vol) hydrogen atmosphere (15).

Cultivation technique. Stirred fermentors with working volumes of 1.0 liter were used for all physiological studies (FL 101, Bio-Tec, Stockholm, Sweden). The pH was controlled with an automatic titrator (Bio-Tec, LP 300) and with 1 M NaOH as the titrant. An autoclavable combined glass reference electrode (GK 4031, Radiometer) was used. Temperature was controlled by means of a thermometer in a stainless steel pocket immersed in the culture, a transistorized relay, and a cartridge heater of 40-W capacity. Temperature was kept constant with an accuracy of ±0.01°C and pH with an accuracy of ±0.05 pH unit. The impeller speed was 200 rpm (Bio-Tec LP 300). An anaerobic atmosphere was maintained by bubbling a mixture of oxygen-free nitrogen and carbon dioxide through the culture at a rate of 0.05 liter/min. The oxygen-free gas mixture was obtained by catalytic reduction of 92% (vol/vol) nitrogen, 5% (vol/vol) carbon dioxide, and 3% (vol/vol) hydrogen. The gas passed through a sterile filter (XX30-025-14; Millipore Corp., Bedford, Mass.) and copper tubing into the culture vessel.

Preparation of inocula. The inocula were prepared from overnight cultures grown under static conditions in bottles of prereduced basal medium containing 1% (wt/vol) glucose (final dry weight, 1.0 mg/ml). The microorganisms were washed once in an anaerobic chamber and resuspended in fresh medium with or without 1% (wt/vol) glucose under anaerobic conditions and then added to the fermentor to an initial dry weight of 0.2 mg/ml.

Determination of bacterial growth. Samples of 10 ml each were taken from the cultures at intervals and centrifuged at 4,000 × *g* for 15 min at 4°C. The supernatant fluid was tested for glucose concentration and enzyme activities. The pellet was washed once in 0.01 M sodium phosphate buffer (pH 7.0) and dried at 110°C for 18 h prior to dry weight determination. All weights were corrected for the weight of the buffer salts (15).

Continuous cultures. The Bio-Tec FL 101 fermentor was used for all experiments. A freshly prereduced medium was pumped into the vessel from a 20-liter reservoir of stainless steel by means of a hose pressure pump (Bio-Tec LP 600) (15). The flow rate could be varied, and the pump was calibrated before the cultivation started. The culture volume was kept constant by an overflow tube in the vessel. The rubber tubing connections were made as short as possible to minimize the risk of air diffusion through the wall. As in the batch experiments, the impeller speed was 200 rpm, and the pH was controlled with an automatic titrator by using 1 M NaOH as titrant. Temperature was controlled at 37°C and pH at 7.0. The medium was inoculated as in batch cultivation. The culture was grown under batch conditions for 12 h, i.e., to the end of the period of active growth, before the continuous feed was started. Samples (10 ml) were taken every 1 or 2 h for dry weight determinations, enzyme assays, and glucose determinations. Samples of the cultures, in the middle and at the end of a 48- to 72-h continuous run, were plated on agar containing horse erythrocytes to determine whether the culture was pure. No contaminants were detected.

Determination of glycosidase activities. α-D-Glucoside glucohydrolase (EC 3.2.1.20), β-D-glucoside glucohydrolase (EC 3.2.1.21), α-D-galactoside galactohydrolase (EC 3.2.1.22), β-D-galactoside galactohydrolase (EC 3.2.1.23), and β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase (EC 3.2.1.30) were assayed according to Nord et al. (14). All enzyme assays were conducted in 0.05 M sodium phosphate buffer (pH 6.2). Hydrolysis of the *p*-nitrophenyl-glucosides was determined spectrophotometrically at 410 nm in a Gilford spectrophotometer 240. One unit of enzymatic activity (U) was defined as the amount of test specimen required to hydrolyze 1 μmol of substrate per min at 37°C. For assay of α-D-glucoside glucohydrolase and β-D-glucoside glucohydrolase, the substrates used were *p*-nitrophenyl-α- and -β-D-glucopyranosides, respectively (5 mM, pH 6.2, 0.5 ml, 2 h). *p*-Nitrophenyl-α- and -β-D-galactopyranosides (5 mM, pH 6.2, 0.5 ml, 2 h) were used for assays of α-D-galactoside galactohydrolase and β-D-galactoside galactohydrolase. *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide (5 mM, pH 6.2, 0.5 ml, 2 h) was used as substrate for β-*N*-acetylglucosaminidase.

Chemical analyses. Glucose was analyzed by the glucose-oxidase method (Glox Kit, Kabi, Stockholm, Sweden). Protein was assayed on culture supernatants after extensive dialysis according to Lowry et al. (11).

Chemicals. All chemicals were of analytical grade unless otherwise stated. The salts and constituents for buffers were obtained from Merck AG, Darmstadt, Germany. *p*-Nitrophenyl-α- and -β-D-glucopyranosides, *p*-nitrophenyl-α- and -β-D-galactopyranosides, and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Choice of strain. The nine strains of *B. fragilis* were cultivated in fermentors under controlled conditions of pH, temperature, gas, and agitation to determine their ability to produce glycosidases. There were great variations in the ability of the nine different strains to produce these enzymes under similar growth conditions (Table 1). In static cultures, all strains showed less than one-tenth of the activities obtained in agitated cultures. Strain B70 was the best producer of α-glucosidase, β-galactosidase, and β-*N*-acetylglucosaminidase activities and was therefore chosen for further studies of the influence of cultural conditions on the production of these enzymes.

Medium composition. *B. fragilis* subsp. *vulgatus* B70 was cultivated in six different prereduced media in stirred fermentors with a working volume of 1 liter. Both the highest yield of cells and the best overall yields of enzyme activities were obtained in the proteose peptone-yeast extract medium (Table 2). The cell yield obtained was lowest in the defined medium in which only very low levels of enzyme activities were found. The peptone-yeast extract medium and the whale meat medium gave similar en-

TABLE 1. *Extracellular glycosidase activities of different strains of B. fragilis grown for 8 to 10 h in stirred fermentors in prereduced proteose peptone-yeast extract medium containing 10 g of glucose per liter at pH 7.0 and 37°C.*

Strain of *B. fragilis*	Bacterial dry wt (mg/ml)	α-Glucosidase (mU/ml)	β-Galactosidase (mU/ml)	β-*N*-Acetylglucosaminidase (mU/ml)
ATCC 8482 subsp. *vulgatus*	1.30	1.2	9.0	6.1
ATCC 8483 subsp. *ovatus*	0.81	41.9	7.8	6.1
ATCC 8503 subsp *distasonis*	1.05	1.6	1.3	7.6
NCTC 9343 subsp *fragilis*	1.55	73.0	26.0	34.0
NCTC 10582 subsp. *thetaiotaomicron*	2.19	3.1	23.0	14.6
B4 subsp. *ovatus*	1.66	93.0	2.4	5.8
B70 subsp. *vulgatus*	2.52	143.8	55.4	133.6
B72 subsp. *vulgatus*	1.50	71.6	9.6	4.9
B153 subsp. *fragilis*	1.32	43.1	4.4	18.9

TABLE 2. *Influence of medium composition on the yield of cells and formation of extracellular glycosidases by B. fragilis subsp. vulgatus B70 in stirred fermentors at pH 7.0 and 37°C*[a]

Medium	Bacterial dry wt (mg/ml)	α-Glucosidase (mU/ml)	β-Galactosidase (mU/ml)	β-*N*-Acetylglucosaminidase (mU/ml)
Prereduced proteose peptone-yeast extract with glucose (10 g/liter)	2.52	143.8	55.4	133.6
Prereduced peptone-yeast extract with glucose (10 g/liter)[b]	2.06	131.5	31.1	128.3
Prereduced tryptone yeast extract with glucose (10 g/liter)[c]	2.46	6.4	10.5	14.8
Prereduced tryptone with glucose (10 g/liter)[d]	1.28	21.8	10.9	7.3
Prereduced defined[d]	0.38	3.7	2.9	1.5
Prereduced whale meat with glucose[e]	1.68	146.5	39.0	80.6

[a] All cultures were harvested 8 to 10 h after inoculation.
[b] Holdeman and Moore (9).
[c] Wahren and Holme (22).
[d] Wahren and Holme (23).
[e] Dalland and Hofstad (1).

zyme activities to the proteose peptone-yeast extract medium but lower cell yields. When tryptone replaced peptone in the medium supplemented with yeast extract, the cell yield was identical to that obtained with the proteose peptone medium, but enzyme activities were much reduced. The proteose peptone-yeast extract medium was therefore chosen for further experiments.

Relationship between bacterial growth and formation of glycosidases. The appearance of glycosidases in the culture fluid during different phases of growth of *B. fragilis* subsp. *vulgatus* B70 was studied in a fermentor of 1-liter volume with prereduced proteose peptone medium containing glucose (10 g/liter) at 37°C and pH 7.0. The period of active growth lasted for about 6 h, followed by a lytic phase. Glucose was the growth-limiting factor in the experiment. Liberation of α-glucosidase, β-galactosidase, and β-*N*-acetylglucosaminidase into the medium increased throughout the experiment (Fig. 1). No β-glucosidase or α-galactosidase was detected in the culture fluid during the 24-h period of cultivation.

Influence of pH on growth and formation of glycosidases. The influence of pH on the yield of cells and glycosidases was tested in the proteose peptone-yeast extract medium (Fig. 2). The highest cell yield (2.5 mg [dry weight] per ml) was obtained at pH 7.0. The pH optimum for production of α-glucosidase (ca. 80 mU/mg [dry weight] of bacteria) and β-*N*-acetylglucosaminidase (ca. 50 mU/mg [dry weight] of bacteria) was at pH 7.0, and for β-galactosidase (ca. 20 mU/mg [dry weight] of bacteria) it lay between pH 6.5 and 7.5. At pH 5.5 and 8.0, only slow growth and low enzyme activities were obtained.

Influence of temperature on growth and formation of glycosidases. The influence of temperature on the yield of cells and glycosidases was tested in the range 31 to 41°C at pH 7.0 (Fig. 3). The maximum cell yield (2.5 mg [dry

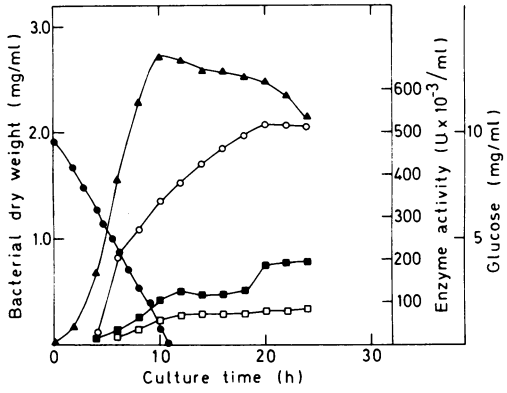

FIG. 1. *Relationship between bacterial growth and formation of extracellular glycosidases of B. fragilis B70. Cultivation was carried out in a stirred fermentor in a prereduced proteose peptone-yeast extract medium at 37°C and pH 7.0.* (▲) *Bacterial dry weight;* (○) *α-glucosidase;* (□) *β-galactosidase;* (■) *β-N-acetylglucosaminidase;* (●) *glucose concentration.*

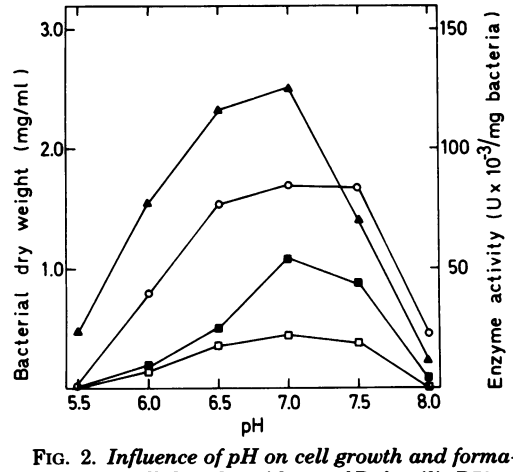

FIG. 2. *Influence of pH on cell growth and formation of extracellular glycosidases of B. fragilis B70 at 37°C in prereduced proteose peptone-yeast extract medium.* (▲) *Bacterial dry weight;* (○) *α-glucosidase;* (□) *β-galactosidase;* (■) *β-N-acetylglucosaminidase.*

weight] per ml) was obtained at 37°C, but the differences between this and the yields at 31 to 39°C were only slight. The enzyme activities were rather similar in the tested temperature range except for α-glucosidase, where the maximal activity was observed at 33°C.

Continuous cultivation. It was desirable for further studies to produce large quantities of glycosidases, and therefore continuous cultivation experiments were carried out. After 12 h, the continuous feed was started with a dilution rate of 0.1 liter/h. The formation of glycosidases remained constant during the whole continuous process (Fig. 4).

DISCUSSION

Glycosidases from gram-positive bacteria such as streptococci (6, 14), pneumococci (20, 23), and *Bacillus* species (17, 19) have been studied extensively during the last few years.

On the other hand, very few reports on glycosidases from gram-negative bacteria, with the exception of *Escherichia coli* (24) and *Pseudomonas* species (2, 8), have been published. A *Bacteroides* amylase was recently studied by McWethy and Hartman (12).

The cell yield obtained in the present investigation by batch cultivation without stirring and pH control was low (0.5 g/liter, dry weight). By using stirred fermentors with pH control, the cell yield was increased about 5 times, and the yield of glycosidases was increased about 10

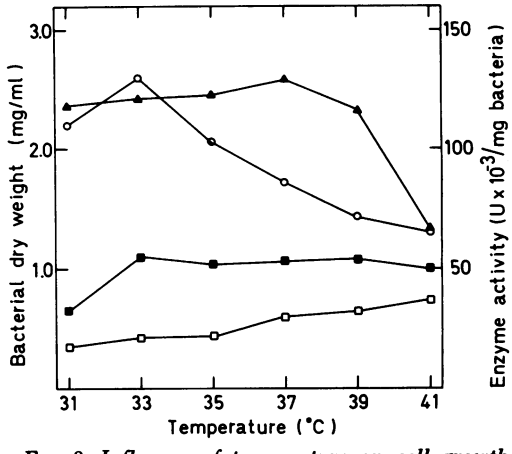

FIG. 3. *Influence of temperature on cell growth and formation of extracellular glucosidases of B. fragilis B70 at pH 7.0 in a prereduced proteose peptone-yeast extract medium.* (▲) *Bacterial dry weight;* (●) *α-glucosidase;* (□) *β-galactosidase;* (■) *β-N-acetylglucosaminidase.*

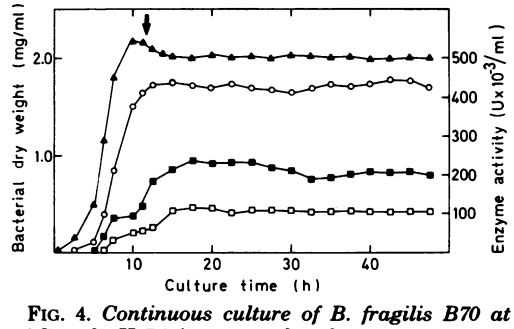

FIG. 4. *Continuous culture of B. fragilis B70 at 37°C and pH 7.0 in a prereduced proteose peptone-yeast extract medium. Arrow indicates start of continuous feed at a dilution rate of 0.1 liter/h.* (▲) *Bacterial dry weight;* (○) *α-glucosidase;* (□) *β-galactosidase;* (■) *β-N-acetylglucosaminidase.*

times. The reproducibility of yields with stirred fermentors was much better than with flask cultures (21).

One of the media, proteose peptone with yeast extract, gave the highest yield of cells and glycosidases. This medium was therefore used in further experiments. Suzuki et al. (19) have also recently shown that proteose peptone-yeast extract medium was more effective than other media for production of glycosidases from *Bacillus thermoglucosidius*. They also observed that proteose peptone selectively promoted glycosidase production.

B. fragilis produced glycosidases both during the logarithmic and the lytic phases. These enzymes are probably released into the medium upon cell lysis, since most glycosidases in gram-negative bacteria are known to be cytoplasm located (2, 8, 18, 24).

Optimal pH for the production of glycosidases from *B. fragilis* was found between pH 6.5 and 7.5. This finding agrees with that of Desai and Goldner (3), who reported maximal β-galactosidase activity in *Streptococcus lactis* at pH 7.0. Kilian and Bülow (10) recently investigated strains of *Enterobacteriaceae* and *Vibrionaceae* for various glycosidase activities and found that pH optima for the different enzymes were between 7.0 and 8.0.

In recent years, techniques have been developed for continuous culture of *B. fragilis* (1, 16). The present study shows that the composition of the medium, pH, and temperature are important factors for optimal growth, and that a strain with high glycosidase activities is needed for production of glycosidases in large amounts. With continuous cultivation of *B. fragilis*, the high yields necessary for the purification of glycosidases are now available.

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