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Defined Conditions for Synthesis of *Bacillus cereus* Enterotoxin by Fermenter-Grown Cultures

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A strain of *Bacillus cereus* produced high levels of enterotoxin when grown in a semidefined medium in a laboratory scale fermenter. The optimum conditions for enterotoxin synthesis by cultures grown in this medium, which contained Casamino Acids and yeast extract, were found to be: inoculation of vigorously growing culture at the 1% level, addition of glucose at a concentration of 1%, control of culture pH at 8.0, incubation at 32°C, use of a moderate stirring rate, and addition of air at low flow rates to minimize foaming. The enterotoxin yield in fermenter-grown cultures was approximately 20 to 50 times higher than the yield obtained in shake flask cultures.

Bacillus cereus has been recognized as the causative agent of a relatively mild form of intestinal illness, involving diarrhea and abdominal pain (6), and also of a more severe illness with vomiting as the primary symptom (8). There is some evidence that the strains responsible for the milder, "classic" food poisoning syndrome can be separated from those causing the more severe symptoms by serotyping flagellar antigens (11).

In recent years *B. cereus* has been shown to produce an extracellular factor that induces fluid accumulation in the ligated ileal loop assay in rabbits (9), evokes a dermal necrotic reaction in guinea pigs (4), and increases vascular permeability in rabbits (5). This factor has been shown to meet two of the three experimental criteria established by Bonventre et al. (1) to determine that a toxin acts directly on the gastrointestinal tract (10) and is therefore referred to as an enterotoxin.

Further investigation into the role of *B. cereus* enterotoxin in the etiology of the food poisoning syndrome(s) awaits production and purification of large quantities of enterotoxin for feeding studies. Up to this time cultures for enterotoxin production had always been grown in complex media in shake flasks. Not only was it difficult to obtain large quantities of toxin by this procedure, but the highly complex culture media required for production of relatively high toxin levels made the task of purification more difficult. It was therefore decided to define the optimal cultural conditions for synthesis of enterotoxin in a laboratory scale fermenter. By employing these conditions, a simplified culture medium could then be used, which would facilitate purification of the enterotoxin. Determination of the optimal conditions for enterotoxin synthesis in laboratory media could also shed light on the conditions required for enterotoxin production in food products.

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MATERIALS AND METHODS

Cultures. Strain B-4ac, which had been isolated from a food poisoning outbreak, was obtained from D. A. A. Mossel, Louvain, Belgium. Strain B-4ac-L was isolated as a colony morphology variant from a nutrient agar (Difco) plate culture of strain B-4ac and was used in all of the experiments described in this paper. Stock cultures were maintained on nutrient agar slants at room temperature. Working cultures were streaked at weekly intervals from stock cultures onto nutrient agar plates, incubated overnight at 32°C, and stored at 4°C.

Growth media. Media included brain heart infusion broth (Difco) supplemented with 0.1% glucose (BHIG) and a medium, designated CA, which was adapted from a medium developed by Evans et al. (2) for the production of *Escherichia coli* enterotoxin. The CA medium was formulated as follows (all quantities in grams per liter): vitamin-free Casamino Acids (Difco), 20; yeast extract (Difco), 6; NaCl, 2.5; K_2HPO_4, 8.71; and trace salts solution, 1.0 ml. The trace salts solution was composed of 5% $MgSO_4 \cdot 7H_2O$, 0.5% $MnCl_2$, and 0.5% $FeCl_3 \cdot 6H_2O$ dissolved in 0.001 N H_2SO_4. Ingredients were dissolved in the order given and the mixture was then adjusted to the desired pH with 5 N NaOH or 5 N HCl. When glucose (10 g/liter, unless otherwise stated) was added aseptically after autoclaving, the medium was designated CAD.

Media were prepared by using glass-distilled water, dispensed into flasks or bottles, and autoclaved at 121°C for 15 min.

Growth conditions. Inocula were prepared by inoculating from a single colony from a working culture into 25 ml of the desired medium in a 125-ml Erlenmeyer flask and incubating overnight (10 to 14 h) at 32°C on a reciprocal shaker. Experimental shake flask cultures were then prepared, using a 0.1% inoculum from the overnight cultures into prewarmed flasks, and were incubated with shaking at 32°C for 5 h. Cultures for fermenter studies were grown by making a 1% transfer from the overnight inoculum into a second flask, incubating 2 h, and inoculating the fermenter medium at the desired concentration (usually 1%) from this flask.

Fermenter studies were performed in a Bioflo Benchtop Chemostat, model C30 (New Brunswick Scientific Co.), using a culture volume of 300 ml. The air supply system consisted of a compressed air tank governed by a Purox CGA-540 two-stage regulator (Union Carbide). Air flow was controlled by a Lab-Crest variable area flowmeter (Fischer and Porter Co.). Dissolved oxygen (DO) was estimated with a New Brunswick model M1016-0208 dissolved oxygen probe, connected to a New Brunswick model DO-50 dissolved oxygen analyzer with recorder. The pH of the culture was monitored with a type pHM 26 pH meter (Radiometer A/S) and was controlled by the addition of 5 N NaOH from a buret regulated by a magnetic valve, model MNV-1 (Radiometer), and a type TTT11 titrator (Radiometer).

Culture growth was followed by determining the optical density at 560 nm (OD_{560}) with a Spectronic 20 colorimeter (Bausch & Lomb, Inc.). Samples were diluted to the linear range with 0.1% peptone water. The OD_{560} was found to be proportional to dry weight.

Standard cultural conditions in the fermenter were as follows: temperature, 32°C; agitation rate, 400 rpm; air flow rate, 944 ml/min (2 standard cubic feet/h); pH controlled at 8.0; inoculation level, 1%; and medium CAD with 1% (wt/vol) glucose.

Preparation of culture supernatant fluids. Culture samples (5 ml) were centrifuged in a Safeguard tabletop centrifuge (Clay-Adams, Inc.) for 10 min at maximum speed. The supernatant liquid was decanted and the pellet was discarded. Unless they were to be tested immediately, supernatant samples were quick-frozen in an ethanol bath and stored at −20°C until used.

Enterotoxin assay. The vascular permeability assay was used as the measure of enterotoxic activity in the supernatant fluid and was performed as previously described (5). Six replicates of each sample were tested in at least two rabbits, and the activity was expressed as capillary permeability factor units administered (CPFUA). All values were standardized with a positive control sample injected into all rabbits.

RESULTS

Shake flask experiments. Preliminary studies using shake flask cultures showed that growth of strain B-4ac-L in CA medium was equal to growth in BHIG medium in both rate and final cell yield. In addition, enterotoxin levels obtained in CA were comparable to those produced in the more complex medium. Similar to results obtained previously with BHIG-grown cultures (5), enterotoxic activity in the culture supernatant fluid reached its highest level after 5 h of growth in CA.

The influence of various cultural conditions on enterotoxin production was first examined with shake flask cultures. A 14-h, stationary-phase culture and a 3-h, exponential-phase culture were each inoculated at 0.1, 1.0, and 10.0% levels into CA. There was no difference among the cultures grown from the young and old inocula in growth rate, final OD_{560} attained, or toxin levels produced in 5-h cultures. However, inoculum size was a very significant factor. Highly toxic filtrates were obtained from cultures that had been inoculated at the 0.1% level. Low enterotoxic activity was detected at 3 h, but none was detected at 5 h in the cultures grown from a 10.0% inoculum. By 2.5 h these cultures had already reached the stationary phase. In contrast, growth of the cultures started from 0.1 and 1.0% inocula did not reach a stationary level until 5 and 4 h, respectively. Final cell yields at 5 h were about the same at all three inoculum levels, but the cultures started with a 10.0% inoculum attained the highest OD_{560}.

Prior to autoclaving, culture flasks containing CA and CAD media were adjusted to various pH values to test the influence of starting pH on enterotoxin production. Cultures grown in CA at an initial pH of 8.0 or 8.5 produced high levels of toxin, but cultures grown at an initial pH of 7.3, 6.5, 6.0, or 5.5 produced less than 15% as much enterotoxin. No toxin was measurable in cultures grown in CAD at a starting pH of 7.0, 7.5, or 8.0, but low levels were present if the starting pH was 8.5. The final pH values of the CAD-grown cultures were found consistently to be between 5.0 and 5.5, but the final pH values of the CA-grown cultures were close to the initial pH. Further studies were undertaken in the fermenter, in which pH could be carefully controlled.

Representative fermenter experiment. Figure 1 demonstrates the time course of growth and enterotoxin production of a culture inoculated at the 1% level into CAD medium in the fermenter. Growth as measured by optical density was exponential for about 3.5 h. The measured DO decreased from the time of inoculation, reaching an undetectable level by 2.5 h. It remained at this level throughout the course of the experiment unless air was introduced at a sufficiently high flow rate. The pH decreased to

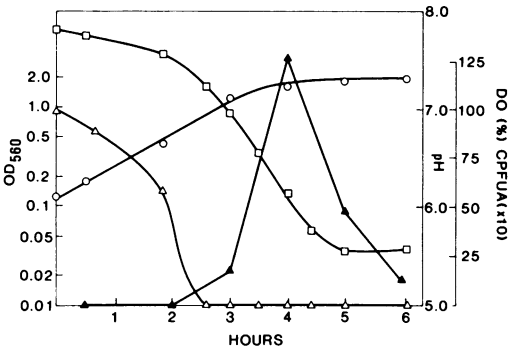

FIG. 1. *Graphic representation of a typical batch experiment in the fermenter. Symbols: ○, OD_{560}; △, DO; □, pH; ▲, enterotoxic activity, expressed as CPFUA.*

a final value of 5.5. It did not increase from this value during the 6-h time period. Enterotoxic activity was first detectable at 2 h. It reached a maximum level at 4 h, at which time the pH of the culture was 6.1. Measurable toxic activity decreased rapidly after 4 h and was at a very low level by 6 h. Samples were routinely taken hourly after 2 h, and experiments were terminated by 6 h.

Influence of glucose concentration. In contrast to the results obtained in the shake flask studies, fermenter-grown cultures produced high levels of toxin in CAD medium. Table 1 lists enterotoxic activity measured in supernatant fluids from cultures grown at varying glucose concentrations. The highest CPFUA was measured in the culture grown with 1% glucose, but relatively high toxin levels were also present in cultures grown without glucose or with 5% glucose. The culture grew more slowly at the 25% glucose concentration, but reached an equivalent cell yield by 7 h of incubation, at which point measured toxic activity was moderate. When the glucose concentration was maintained at 1% throughout the experiment, toxic activity reached a peak at 4.5 h and remained high through 9 h, at which time the experiment was terminated.

Effect of inoculum size. In shake flask studies, too large an inoculum precluded toxin production. However, under controlled cultural conditions in the fermenter, cultures inoculated at the 10% level produced relatively high enterotoxin titers. Higher levels of enterotoxic activity were measured in cultures inoculated at lower concentrations (Table 2).

Influence of culture pH. The results presented in Table 3 indicate that pH control was an important factor in enterotoxin production. Measured enterotoxic activity was approximately doubled in cultures grown at a constant pH of 7.5 or 8.0 over the levels detected in cultures grown at pH 6.5 or 7.0 or with no pH control. Enterotoxin production decreased sharply in cultures grown at pH 6.0 and 8.5. No toxin was detectable in cultures grown below pH 6.0.

Temperature effects. Incubation temperature was varied to relate growth rate to enterotoxin production (Table 4). The culture growth rate increased with increasing temperature, and the time at which the maximum enterotoxic activity was measured decreased with increasing growth rate. However, enterotoxin production was not correlated with growth rate, but a very striking maximum in measured toxic activity was observed in cultures grown at 32°C.

Effect of aeration conditions. Oxygen availability to the growing bacteria can be influ-

TABLE 1. *Enterotoxin production in fermenter-grown cultures in CAD as influenced by glucose concentration*

Glucose (%)	Maximum CPFUA obtained	Time (h) of maximum CPFUA
0	10.3	5
1	20.5	5
5	12.4	5
25	7.1	9
Maintained at 1 ± 0.5	12.2-21.7[a]	4.5-9

[a] Range of values obtained over the times listed.

TABLE 2. *Enterotoxin production in fermenter-grown cultures in CAD as influenced by inoculum size*

Inoculum (%)	Maximum CPFUA obtained	Time (h) of maximum CPFUA
1	28.2	5
5	27.5	4
10	13.3	4

TABLE 3. *Enterotoxin production in fermenter-grown cultures in CAD at controlled pH*

pH	Maximum CPFUA obtained	Time (h) of maximum CPFUA
Uncontrolled	12.7	4
Controlled at:		
8.5	6.5	5
8.0	28.2	5
7.5	24.9	5
7.0	16.8	5
6.5	10.7	5
6.0	4.4	5
5.5	0	
5.0	0	

TABLE 4. *Growth and enterotoxin production in fermenter-grown cultures in CAD at various temperatures*

Temp (°C)	Generation time (min)[a]	Maximum CPFUA obtained	Time (h) of maximum CPFUA
20	140	1.7	8.5
25	60	11.8	7.5
32	50	20.5	5
37	48	4.8	4.5
42	38	3.2	4

[a] Determined graphically from the exponential growth phase.

enced by the efficiency of mixing in the vessel and by the flow rate and composition of the gas being supplied.

The standard agitation rate of 400 rpm, originally chosen because this rate afforded good mixing without the impeller becoming unstable, was found to be the optimum rate for enterotoxin production (Table 5). At 200 rpm mixing was poor, and at the higher agitation rates very large quantities of foam were generated. The reason for the exceptionally low toxin level generated in cultures agitated at 600 rpm remains obscure.

The data presented in Table 6 indicate that low air flow rates favor enterotoxin production. The minimum air flow rate attainable with the air supply system present in the model C30 chemostat was 190 ml/min, but this could not be well regulated. When an independent air supply system was employed that could precisely control lower air flow rates, a dramatic increase in measured enterotoxic activity was observed. The degree of foaming of the culture was also greatly decreased when lower air flow rates were employed.

The DO level was also varied (Table 7). Although lower levels of toxin were produced under anaerobic conditions than under aerobic conditions, this is the first time that toxin production has been observed in an oxygen-free environment. Growth was somewhat slower than in the presence of air, but a high final cell yield was obtained. The addition of nitrate as an electron acceptor for the respiratory chain increased the growth rate slightly, but did not improve toxin production. Anaerobic conditions were maintained by flushing the vessel with N_2 before inoculation and by bubbling N_2 through the growing culture at a rate of 50 ml/min.

The DO could not be maintained at a constant level by pumping in air, but was kept above zero by greatly increasing the air flow rate. Under these conditions toxin production was very low. In contrast, supplying pure oxygen at a rate of 50 ml/min maintained the DO concentration at >85% of maximum solubility in equilibrium with pure oxygen (i.e., four to five times the maximum solubility in equilibrium with air) and yet allowed production of high levels of enterotoxin.

DISCUSSION

The optimum conditions for synthesis of *B. cereus* enterotoxin by a fermenter-grown culture have been established in the course of this study. These conditions are: (i) inoculation of a vigorously growing culture at the 1% level, (ii) addition of glucose to the medium at a concentration of 1%, (iii) control of culture pH at 8.0, (iv) incubation at 32°C, (v) use of a moderate

TABLE 5. *Influence of agitation rate on enterotoxin production in fermenter-grown cultures in CAD*

Agitation rate (rpm)	Maximum CPFUA obtained	Time (h) of maximum CPFUA
200	11.1	5
400	20.5	5
600	5.8	5
800	14.8	4

TABLE 6. *Influence of air flow rate on enterotoxin produciton in fermenter-grown cultures in CAD*

Air flow rate (ml/min)	Maximum CPFUA obtained	Time (h) of maximum CPFUA
0	66.0	5.5
20	51.1	5.5
50	100.4	5
100	34.4	5
300	69.0	5
944	20.5	5

TABLE 7. *Influence of gas composition on enterotoxin production in fermenter-grown cultures in CAD*

Gas	DO maintained[a]	Maximum CPFUA obtained	Time (h) of maximum CPFUA
N_2	0	22.9	6
N_2[b]	0	12.7	5.5
Air	>0[c]	11.0	4
Air	>10[c]	6.4	4
O_2	>85	62.1	6

[a] The DO is expressed as percent saturation of oxygen in the liquid phase under operating conditions. The 100% level was set with air for the N_2 and air experiments and with pure O_2 for the O_2 experiment.

[b] With addition of 1% KNO_3 to the medium.

[c] The DO was maintained as high as possible by repeatedly increasing the air flow rate to 1,640 ml/min.

stirring rate (400 rpm), and (vi) addition of air at low flow rates.

Since enterotoxin is synthesized during the exponential growth phase, it is likely that the use of low inoculum levels increased the level of toxin produced by prolonging the length of the exponential growth phase of the culture. The addition of glucose could exert the same effect by inhibition of the onset of sporulation (7). The influence of glucose is probably twofold: enhancement of exponential growth and inhibition of protease synthesis, a sporulation-related event. The presence of significant levels of protease in supernatant fractions is likely responsible for the previously reported (10) instability of enterotoxic activity.

Control of culture pH in a rather narrow range doubled the enterotoxin yield. We believe that it is enterotoxin synthesis, not stability, that is pH sensitive, since Spira and Goepfert (10) reported that preformed enterotoxin is stable in the pH range 5.0 to 10.0.

The failure of enterotoxin production to parallel increases in growth rate at temperatures above 32°C may be related to the length of the exponential growth phase in these cultures. The very rapidly growing cultures may have reached stationary phase before producing significant levels of toxin. Alternatively, a step in toxin synthesis may be heat labile. Previous work (5) has shown that preformed toxin is stable at 45°C, provided proteolytic attack is prevented.

The degree of culture agitation or foaming influenced the level of measured toxic activity more than did the level of available oxygen. Toxic activity was higher in cultures provided with either pure oxygen or air at low flow rates than in those with air at high flow rates. High gas flow and agitation rates greatly increased the degree of foaming in the medium. Oxygen transfer rates in the culture medium are much higher in the fermenter than they are in shake flasks (3). In a related series of experiments using shake flask cultures (unpublished data), we found that changes which increased oxygen transfer rates, which included use of faster shaker speeds, smaller culture volumes, and baffled flasks, resulted in greater foaming but lowered enterotoxin recovery. We believe that excessive foaming inactivates preformed toxin, as observed by William Spira (Ph.D. thesis, University of Wisconsin, Madison, 1974).

Enterotoxin production under truly anaerobic conditions was observed for the first time in this study. This must be taken into account when formulating theories concerning the mode of action of *B. cereus* food poisoning. Until now, it had been assumed that preformed toxin had to be ingested in order to elicit food poisoning symptoms. If the observed illness could be caused either by preformed toxin or by toxin synthesized by cells growing in the intestine, this could account for the disparity of onset times observed in certain outbreaks (10). The possibility of toxin synthesis by cells growing in the intestine had also been dismissed because it is known that the relatively low counts of *B. cereus* generally found in many foods cannot compete well with the normal intestinal flora. However, the large numbers of organisms present in mishandled food might survive and grow long enough in the intestine to produce significant toxin levels.

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