FACTORS INFLUENCING ENDOSPORE FORMATION IN THE GENUS BACILLUS

by

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INTRODUCTION

The aerobic and anaerobic sporeformers, which constitute the genera Bacillus and Clostridium, are abundant in nature, and therefore commonly occur as contaminants in a wide variety of substances. The distinctive feature of these genera---the formation of endospores---has caused a widespread interest in these organisms. Although considerable early experimental work was published in the nineteenth century on endospore formation, the actual stimulus causing an organism to form endospores is still not known. The problem remains a question of importance, both from a theoretical and from a practical point of view. The importance of these organisms in the industrial preservation of foods has stimulated extensive studies of the factors involved in the resistance of endospores to chemical and physical agencies, particularly heat.

From a theoretical standpoint, the cytological changes and metabolic processes that occur during the process of endospore formation are of great interest. In general, factors which cause a stimulation or suppression of endospore formation act at the same time to stimulate or suppress growth to a greater or lesser extent. Since endospore formation is associated so closely with optimum growth conditions, it is difficult to differentiate between factors which cause a stimulation in growth of an organism and factors which stimulate endospore formation. The present study was undertaken to clarify the relationship

of optimum growth conditions for certain species of the genus Bacillus to the rate and time of endospore formation.

REVIEW OF LITERATURE

Since 1890, when Buchner (1) stated that the degree to which a medium favors endospore production is reflected by the percentage of endospores formed, or the "intensity of spore formation", percentage of sporulating cells has been used extensively to express the effect of a medium upon endospore formation. Later authors have extended this to include some measure of the total number of cells present at a corresponding time interval.

Knaysi (2) noted that as the time interval used in measuring the effects of two media on endospore formation increased, the percentage of endospores tended to become equal. In addition, cell disintegration occurrs during an extended time interval. The practice of reporting percentage of endospores was considered by Knaysi to be misleading, because it assumes that the vegetative cells present in the culture at a given moment represent all the cells that did not sporulate. Therefore, he recorded his results in terms of absolute number of endospores and vegetative cells per milliliter at any given time.

Henrici (3) found that Bacillus coherens proceeded to endospore formation more rapidly on dilute agar slants and more

rapidly in heavily-seeded than in lightly-seeded cultures. He recorded his results as percentage of free endospores among 200 cells counted at each time period. Brunstetter and Magoon (4) inferred from this that Henrici's data showed that the rate of endospore formation was determined not by the concentration of the cells alone but by the ratio of the population density to the concentration of nutrient material.

Williams (5) found that the percentage of endospores of Bacillus subtilis varied inversely with the amount of peptone in the medium, and recorded the percentage of spores among 500 cells counted.

Essentially the same results were recorded by Brunstetter and Magoon (4). Using a strain of Bacillus mycoides, they found that the percentage endospores, as determined after 24 hours incubation, increased as the concentration of the peptone decreased. On the other hand, growth, as measured by the amount of sediment in the aerated tubes after centrifugation, was proportional to the amount of peptone present.

Foster and Heiligman (6) reported that the inclusion of two milligrams per liter of glucose in an amino acid medium increased the total count of Bacillus cereus by only 23 percent but increased sporulation by 2500 percent. The growth was expressed as total count per milliliter after 72 and 96 hours' incubation. The percentage endospores present was calculated from a count of 400 cells at each time interval. These authors concluded that "the glucose evidently exerts an effect specifi-

cally on sporulation.....".

Henrici (3) noted that with Bacillus megatherium grown on nutrient agar slants "spore formation commenced practically at the point of inflection between the logarithmic growth phase and the resting phase." In summarizing data published prior to 1931, Cook (7) concluded that "the experiments in which substances are added to the media show definitely that the process (of sporulation) must be correlated in some way with the growth of the organisms."

Roberts and Baldwin (8) found that agar in concentrations as low as 0.06 percent increased sporulation of Bacillus subtilis. The percentage sporulation of this organism in vigorously aerated Bacto-peptone broth was considerably less than in unaerated medium made slightly viscous with agar. Larson, Cantwell, and Hartzell (9) found that below a surface tension of 45 dynes Bacillus subtilis grows at the bottom of the tube, but no endospores were formed. He concluded that oxygen requirement was not, therefore, the main reason for surface growth.

With aerated peptone broth, Brunstetter and Magoon (4) found no endospores after 24 hours' aeration with B. brevis, B. circulans, B. mesentericus, B. laterosporus, B. subtilis, and other organisms of this same genus. Bacillus mycoides responded to aeration, and an increased rate of aeration increased the percentage of endospores. They also found that the number of spores relative to the number of vegetative cells increased as

the amount of peptone present in the culture decreased.

EXPERIMENTAL

Description of Cultures

Thirty-two cultures of the genus Bacillus were collected from various sources. Some were old stock cultures, while others were isolations from soil, flour, fruits, and oleomargarine. All cultures were inoculated on one percent glycerol agar to determine the presence or absence of vacuoles in the cell cytoplasm. This characteristic was used with measurements of cell width on nutrient agar and size of endospores to separate the species. Casein hydrolysis, the reduction of nitrates to nitrites, gelatin hydrolysis, starch hydrolysis, the utilization of citrate, and the production of acetylmethylcarbinol were determined. Fermentation studies were made using xylose and arabinose on an ammoniacal nitrogen medium and dextrose, sucrose, and lactose in nutrient broth fermentation tubes. All media and tests were made according to methods described by Smith, Gordon, and Clark (10), whose work was used as a basis for identification of organisms. The species and strains studied are shown in Table 1.

The growth requirements of the genus Bacillus have not been extensively studied. Biotin has been reported to be required for B. mesentericus (11), B. coagulans (12), B. mycoides (10),

Table 1. Comparison of cultures on amino acid and on ammonium salts media.

Culture and strains studied	Amino acid medium						Ammonium salts medium					
	- biotin			+ biotin			- biotin			+ biotin		
	Days											
	1	2	7	1	2	7	1	2	7	1	2	7
B. cereus.....#1	-	-	+	+	+	+	-	-	-	-	-	-
.....#7	+	+	+	+	+	+	-	-	-	-	-	-
.....#12	-	-	+	+	+	+	-	-	-	-	-	-
.....#13	+	+	+	+	+	+	-	-	-	-	-	-
.....14	+	+	+	+	+	+	-	-	-	-	-	-
.....#17	+	+	+	+	+	+	-	-	-	-	-	-
B. cereus var. mycoides.....#3	-	-	+	+	+	+	-	-	-	-	-	-
B. megatherium.....#2	+	+	+	+	+	+	+	+	+	+	+	+
....#31	+	+	+	+	+	+	+	+	+	+	+	+
B. laterosporus....#4	-	-	-	-	-	-	-	-	-	-	-	-
B. subtilis.....#5	+	+	+	+	+	+	+	+	+	+	+	+
.....#26	+	+	+	+	+	+	-	+	+	-	+	+
.....#27	+	+	+	+	+	+	-	+	+	+	+	+
.....#28	+	+	+	+	+	+	-	+	+	-	+	+
.....#30	+	+	+	+	+	+	+	+	+	+	+	+
.....#32	+	+	+	+	+	+	+	+	+	+	+	+
B. pumilis.....#6	+	+	+	+	+	+	+	+	+	+	+	+
.....#8	+	+	+	+	+	+	+	+	+	+	+	+
.....#10	+	+	+	+	+	+	+	+	+	+	+	+
.....#11	+	+	+	+	+	+	-	+	+	+	+	+
.....#16	+	+	+	+	+	+	-	+	+	+	+	+
.....#18	+	+	+	+	+	+	-	+	+	+	+	+
.....#25	+	+	+	+	+	+	+	+	+	+	+	+
B. coagulans.....#9	+	+	+	+	+	+	+	+	+	+	+	+
B. brevis.....#15	+	+	+	+	+	+	-	+	+	+	+	+
.....#22	+	+	+	+	+	+	-	+	+	+	+	+
B. alvei.....#19	+	+	+	+	+	+	-	+	+	+	+	+
B. sphaericus.....#21	+	+	+	+	+	+	-	-	-	-	-	-
B. sphaericus var. fusiformis...#20	+	+	+	+	+	+	-	-	-	-	-	-
B. polymyxa.....#24	+	+	+	+	+	+	+	+	+	+	+	+
B. firmus.....#29	+	+	+	+	+	+	+	+	+	+	+	+
B. lentus.....#33	+	+	+	+	+	+	+	+	+	+	+	+

\+ = Visible turbidity
\- = No visible turbidity

B. polymyxa (13), and B. macerans (13). As more information on the biotin requirements of these organisms was desired, a comparison of all the cultures was made on an ammonium salts and on an amino acid media, with and without added biotin. The amino acid medium was used by Glinka-Tschernorutzky (14) for cultivation of Bacillus mycoides, but growth of other species of Bacillus on this medium has not been reported. The medium was composed of 0.1 percent di-potassium phosphate, 0.05 percent magnesium sulfate, 0.01 percent sodium chloride, 0.002 percent ferrous sulfate, 0.01 percent calcium chloride, 0.005 percent of glycine, d-valine, dl-alanine, phenylalanine, l-aspartic, l-leucine, and d-arginine, and 0.0025 percent l-cystine, l-tryptophan, l-tyrosine, l-proline, l-histidine monohydrochloride, d-lysine dihydrochloride, and dl-serine. One percent glucose was also added to serve as an additional energy source, although it was not absolutely essential. The ammonium salts medium consisted of 0.5 percent mono-ammonium phosphate, 0.03 percent magnesium sulfate, 0.01 percent mono-potassium phosphate, 0.016 percent di-potassium phosphate, and 1.0 percent glucose. The tubes were made up in duplicate and one microgram of biotin per 100 milliliters was added to one-half the tubes before autoclaving. The media were adjusted to pH 7.0 and autoclaved at 15 pounds pressure for 20 minutes. The cultures were incubated at 37° C. and examined for growth and endospore formation at the end of 1, 2, and 7 days. Results are shown in Table 1.

The added biotin had no effect on species which did not

grow on the amino acid or the ammonium salts media. With several species it apparently accelerated the rate of growth of the organisms. All cultures in the amino acid medium which showed visible turbidity were found to contain free endospores or sporangia upon microscopic examination. Only two of the cultures, Bacillus megatherium (#2) and Bacillus subtilis (#26), showed the presence of endospores in the ammonium salts medium.

Four serial transfers in a 10 day period were made into the amino acid and the ammonium salts medium with Bacillus megatherium (#2) and Bacillus subtilis (#5 and #23). The last tube in the series was held six days and then the tubes were examined for endospore formation. In the amino acid medium, all three cultures consisted almost entirely of free endospores, while on the ammonium salts medium vegetative cells were numerous but no endospores were present.

Materials and Methods

Aeration was carried out in four-liter flasks which contained two liters of standard nutrient broth. The compressed air hose was connected to a series of four liter bottles filled with water to eliminate drying. The hose was then attached to a length of six millimeter glass tubing which extended through the cotton plug to the bottom of each flask. The air was bubbled through the flasks as vigorously as possible without causing the foam to reach the cotton stopper. Since Knaysi (2)

showed that the percentage sporulation in the foam was considerably greater than in the liquid, samples were taken with a sterile 10 milliliter pipette from the center of the liquid. Any foam adhering to the sides of the pipette was mechanically removed with cotton. A small amount of liquid was drawn into the pipette and blown out before the sample was taken to insure the removal of any endospores picked up from the foam.

The standard nutrient broth was composed of 1.0 percent Difco bacto-peptone and 0.3 percent Difco bacto-beef extract. The pH was adjusted to 7.0 and the flasks were autoclaved at 15 pounds pressure for one hour. The flasks were incubated at 30° C.

The inoculum was a cell suspension made by washing five to seven day-old agar slant cultures with sterile distilled water. The suspension was heat-shocked in a water bath at 70° C. for 10 minutes to kill any remaining vegetative cells. Thus all cells counted in the growth curve were due to germination of the endospores and were not vegetative cells carried over as the inoculum itself. No attempts were made to keep the size of the inoculum constant, since it was felt that aeration experiments carried out at different times could not be compared directly.

Since for this work it was desirable to have a method of direct microscopic counting which did not have to be counted at the same time the sample was made, a modification of Henrici's technique (3) was used. A standardized platinum loop, calibrated to 0.01 milliliter was used in place of a pipette to

spread the material on the slide over an area of one square centimeter. Three square centimeters were smeared at each time interval, and 100 representative fields were counted on the three smears. As the results indicated that a count of only 60 fields might be sufficiently accurate, a comparison of counts on 60 and 100 fields was made. The percentage error from counts of 60 fields as compared with that of a 100 fields is shown in Table 2. In all succeeding counts, an average of 60 fields was taken, which was reduced to 10 fields in subsequent counts where turbidimetric readings were made concurrently to check the accuracy of this method of direct counting.

One to one hundred dilutions of the sample were made in a sterile 99 milliliter water blank and direct counts were made from this dilution when the growth became too great to count accurately. All counts from 1 to 100 dilutions are marked in the tables as such. A comparison of direct counts of undiluted and diluted samples was made on Bacillus subtilis (#28), as shown in Tables 21 and 22, Appendix, and Fig. 10. Although the 1 to 100 counts were appreciably higher than the counts on the undiluted sample, the curves are almost identical. The growth curves shown were obtained by plotting the logarithm of the number of bacteria per milliliter against the hours of incubation.

The amount of broth carried over on the platinum loop and the relative ease with which the material spread on the slide varied with the amount of growth in the flask. Early smears did not adhere well to the loop or slide, while smears made at

later time intervals were sometimes viscous, resulting in a greater amount of broth being transferred to the slide. These factors introduced an unavoidable error. One to 100 dilutions were also much more difficult to spread over the slide. When varying agar concentrations were present in the nutrient broth, the medium spread uniformly over the slide even in the early counts.

Except when otherwise indicated, the slides were stained with Hucker's gentian violet for 30 seconds, washed gently, and allowed to air dry. A spore stain was used only in cases where vacuolation was so great as to cause some doubt as to the early stages of endospore formation. The simple stain was preferred to a more complicated staining method since the harsh treatment in most spore stains would wash off many of the organisms and cause an error in the direct count. Although a cleared area was present in the cells preceding endospore formation, the cell was not considered a sporangium until the endospore was clearly formed and became refractive.

To provide another method of estimating growth, turbidimetric readings were made on the samples at the same time as the direct count on several of the aeration experiments. Turbidimetric readings were taken on a Coleman universal spectrophotometer at a wave length of 500 millimicrons. A calibration curve was set up for each strain of Bacillus cereus (#1 and #7) by plotting the logarithm of the percentage transmittance against varying dilutions of the bacteria. A turbidimetric

reading and a direct count with a Petroff-Hausser counting chamber were made on one dilution, so that turbidimetric readings could be read directly from the curve as millions of bacteria per milliliter.

Not only does the turbidimetric method depend upon the amount of substance in suspension, but also on the size and shape of the suspended particles, their relative opacity or transparency, the relation between particle size and the wave length of light used, and the uniformity with which a given turbidity may be reproduced (15). The turbidity readings could be reproduced accurately when more than one reading was taken at the same time interval with a different sample. There was some variation in the size of the organisms at different phases of the growth curve. There was also a possibility that the change of the organism from a vacuolated to a granular state as endospore formation proceeded might cause some variation in readings. However, the growth curves from the direct counts and the turbidimetric readings were very similar, as shown by comparing Figs. 3 and 4 and Figs. 5 and 6.

The percentage endospore formation in this work was arbitrarily defined as the percentage of cells present as sporangia among the total cells counted. This definition was adopted as the aeration was stopped when the majority of the cells were present as sporangia. This was done since this study dealt, for the most part, with the early stages of endospore formation.

Preliminary Studies

Effect of Added Colloids in Unaerated Nutrient Broth. To determine the relative amount of growth and approximate time of endospore formation in unaerated nutrient broth, a series of 150 milliliter Erlenmeyer flasks containing 100 milliliters of nutrient broth was used. One of the following colloids was added to a series of flasks in the indicated concentrations: 0.5 percent Bacto-gelatin, 0.5 percent vitamin-test casein, five milliliters of one and one-half percent solution of colloidal clay, 0.06 percent agar agar, 0.3 percent agar agar. One flask of nutrient broth served as a control. After addition of the colloids, the pH of each flask was adjusted to 7.0 and autoclaved at 15 pounds pressure for 20 minutes. The inoculum was a heat-shocked suspension of Bacillus cereus (#1).

On the direct microscopic counts, an average of 10 microscopic fields was enumerated at each time interval. Growth was stopped when the majority of the cells were present as sporangia, or for a maximum period of 50 hours. The number of cells per milliliter at each time interval for the nutrient broth control, the one and one-half percent colloidal clay, the 0.5 percent gelatin, the 0.5 percent casein, the 0.06 percent agar, and the 0.3 percent agar are shown in Tables 3, 4, 5, 6, 7, and 8, Appendix.

The nutrient broth control (Table 3, Appendix) and the flasks containing added colloidal clay (Table 4, Appendix) and

gelatin (Table 5, Appendix) showed no appreciable increase in numbers from the time the first count was made following 10 hours incubation until they were discarded. Endospore formation did not occur. In the nutrient broth flask containing added casein (Table 6, Appendix) growth was also slight, although free endospores were noted between 16 and 20 hours. Free endospores were very few in number and were not recorded, the percentage endospore formation being calculated as the relative number of cells present as sporangia.

The addition of 0.06 percent agar to the nutrient broth (Table 7, Appendix) caused a slight increase in growth and after 20 hours incubation many cells showed areas which did not stain intensely, but sporangia were never present. In the flask containing 0.3 percent agar (Table 8, Appendix) growth was particularly heavy in the top three centimeters of the flask, from which the samples were taken. At the 40-hour sampling, a few free endospores were present.

It is probable that if the flasks had been held over for longer periods of time most of them would have contained free endospores. However, it was apparent that stationary cultures were not suitable for observing early stages in endospore formation.

Aeration of Cultures. The amount of oxygen available to an organism is known to be a limiting factor in endospore formation (16, 17). Although the influence of oxygen on endospore formation will not be considered in this work, it was desirable

to use a method which would supply adequate oxygen to give maximum growth and endospore formation in a relatively short time interval. For this purpose a series of preliminary studies were made with nine stock cultures to determine the effect of aerating by bubbling compressed air through flasks of nutrient broth. The procedure was identical to that described with unaerated nutrient broth, except that a piece of four millimeter glass tubing extended to the bottom of the flask and was connected to the compressed air hose.

After 24 hours incubation, two strains of Bacillus cereus (#1 and #7) were composed almost entirely of sporangia. The remaining cultures, B. pumilis (#6 and #18), B. alvei (#19), B. sphaericus (#21), B. sphaericus var. fusiformis (#20), B. lentus (#33), and B. laterosporus (#4), showed no endospore formation.

Since the two strains of Bacillus cereus responded to aeration, they were used in succeeding aeration experiments. The amount of growth and percentage endospore formation obtained by aeration of Bacillus cereus (#1) are shown in Table 9, Appendix.

There was a marked increase in growth and endospore formation using this method of aeration, as compared with this same organism on unaerated nutrient broth. The culture grew so rapidly when aerated that it was impossible to get accurate counts for time intervals after 18 hours. Therefore, the number of cells per milliliter past that time are not shown, but percentage endospore formation at succeeding time intervals was

determined. At 20 hours, 20 percent of the cells contained cleared areas, and these were clearly formed endospores at 22 hours.

Aeration Experiments

Comparison of Growth in Nutrient Broth and in One Percent Peptone. Standard nutrient broth and one percent Difco bacto-peptone flasks were aerated simultaneously to observe the relative growth rates and percentage endospore formation of Bacillus cereus (#1) in the two media. The same amount of inoculum was added to both flasks. Table 2 shows the number of cells per milliliter and the percentage endospores in nutrient broth, and includes the percentage error of the average of counts from 60 fields as compared to 100 fields. The nutrient broth growth curve is shown in Fig. 1. Growth and endospore formation present in the one percent peptone broth is shown in Table 10, Appendix, and the growth curve in Fig. 2. The number of bacteria per milliliter for the growth curves shown in Figs. 1 and 2 was taken from the average count for 60 fields.

Samples were taken at intervals from 1 to 10 hours to show the early stages of growth, and from 24 to 35 hours to show the growth rate after endospore formation had commenced. In the samples which were taken in the early stages of growth, the rods were large and took the stain uniformly, while in the later samples the rods were much shorter and adsorbed the dye less

Table 2. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation in aerated nutrient broth, including percentage error from 100 fields on direct counts.

Hours	Log counts per ml	% error on 60 fields as compared with 100 fields	Number of cells per ml	Percentage spore formation
0.42	5.0734	14.6	1.18×10^5	0.0
0.92	5.3814	15.2	2.41×10^5	0.0
1.42	5.3900	16.3	2.46×10^5	0.0
1.83	5.1625	0.0	1.45×10^5	0.0
2.83	5.4459	7.0	2.79×10^5	0.0
3.59	5.9658	6.4	9.24×10^5	0.0
4.08	6.3849	0.8	2.43×10^6	0.0
5.08	6.2165	7.3	1.65×10^6	0.0
6.08	6.7363	0.0	5.45×10^6	0.0
10.93	6.9886	-	9.74×10^6	0.0
24.83	7.1244	-	1.33×10^7	91.0
26.08	7.0084	3.4	1.02×10^7	91.0
28.92	7.1154	11.2	1.30×10^7	88.7
31.42	7.1403	17.4	1.38×10^7	92.9
35.42	7.1511	5.8	1.42×10^7	94.8

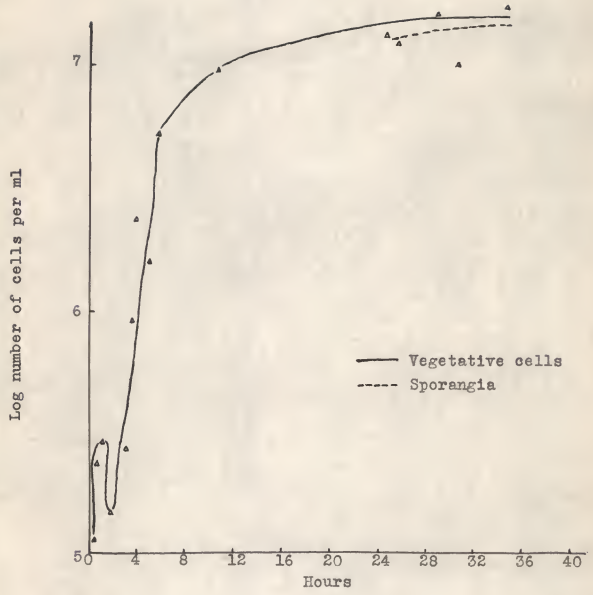

Fig. 1. Bacillus cereus (#1). Growth curve in aerated nutrient broth.

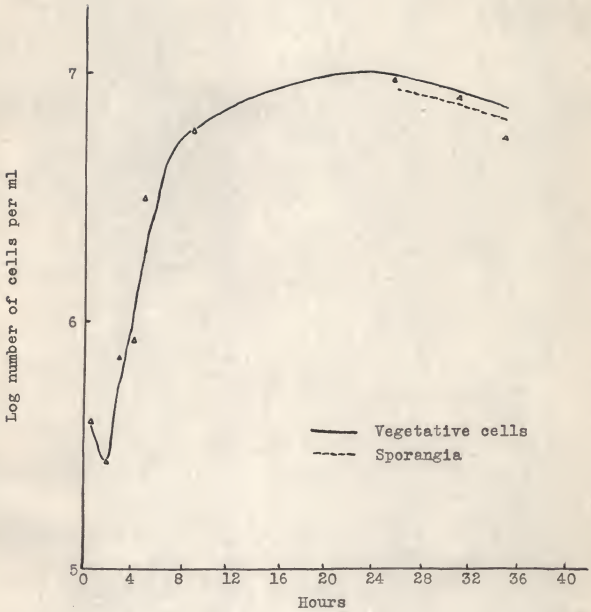

Fig. 2. Bacillus cereus (#1). Growth curve in aerated one percent peptone.

intensely, with the endospore filling approximately one-half of the cell.

Effect of Light and Heavy Inocula on Growth and Endospore Formation. In order to show the effect of light and heavy inocula on all phases of the growth curve, two identical runs were made. Samples were taken at frequent intervals from 6 to 12 hours on the first run, and from 12 to 40 hours on the second run. The inoculum was Bacillus cereus (#1). The light inoculum consisted of 1 milliliter of a spore suspension, and the heavy inoculum was 20 milliliters of the same suspension.

Gray's method of spore staining (18) was used on the direct counts, as this method gave a maximum differentiation of endospores and vegetative cells with no more washing and drastic staining methods than was required for a simple stain. Turbidimetric readings were taken, so only an average of 10 fields was made on the direct counts. An acid-fast stain (19) was made on duplicate slides in the early stages of endospore formation to confirm Gray's method of staining. In Gray's method, very young spores adsorbed the stain lightly, so counts on endospores were taken from the time when they were clearly distinguishable on the acid-fast stain.

The number of cells per milliliter and percentage endospore formation during the early stages of growth are shown in Table 11, Appendix, and the growth curve from the direct count in Fig. 3. For the heavy inoculum during early stages of growth, the number of cells per milliliter and percentage endospore formation

are shown in Table 12, Appendix, and in Fig. 3. The turbidimetric readings for both the light and heavy inocula for the same intervals are recorded in Table 13, Appendix, as millions of cells per milliliter, and the growth curves are shown in Fig. 4.

The number of cells per milliliter and the percentage endospore formation for the light inoculum during the latter stages of growth (12 to 40 hours) are shown in Table 14, Appendix, and the growth curve in Fig. 5. Similar data for the heavy inoculum are shown in Table 15, Appendix, and in Fig. 5. The turbidimetric readings at these same time intervals for both the light and heavy inoculum are shown in Table 16, Appendix, and in Fig. 6.

Though there were differences in total numbers with the light and heavy inocula, they were relatively slight, and varied on the two runs. For the shorter, 6 to 12 hour, incubation periods the heavy inoculum gave higher values than did the light inoculum. The reverse was true on the 12 to 40 hour incubation periods, although the inoculum was the same on both runs and conditions were duplicated as closely as possible.

On the samples taken following 6 to 12 hours growth, the cells gradually decreased in length, but no vacuoles were present at 12 hours even though the cells did not take the stain as intensely as the young cells. Vacuoles were still present at 15 hours. After 18 hours endospores were very distinct, and were present in most of the cells.

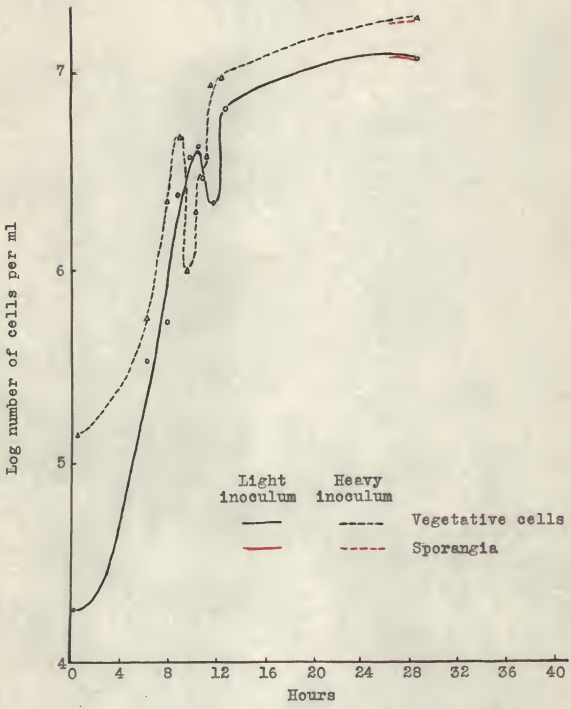

Fig. 3. Bacillus cereus (#1). Growth curve with both light and heavy inocula during early stages of growth.

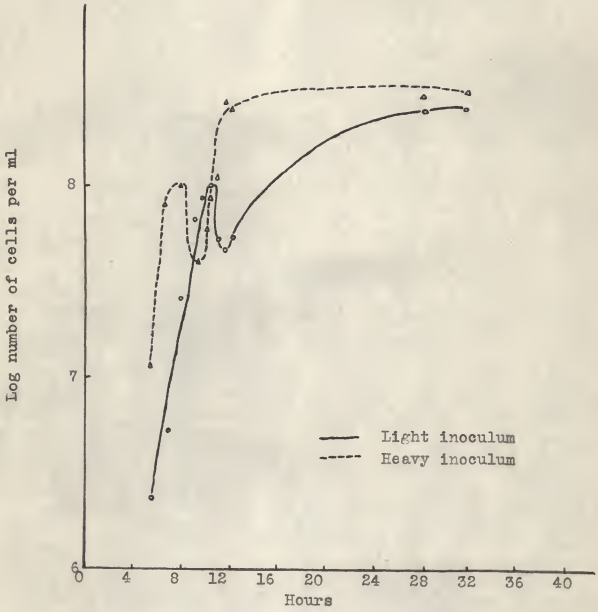

Fig. 4. Bacillus cereus (#1). Growth curve with both light and heavy inocula during early stages of growth, as measured turbidimetrically.

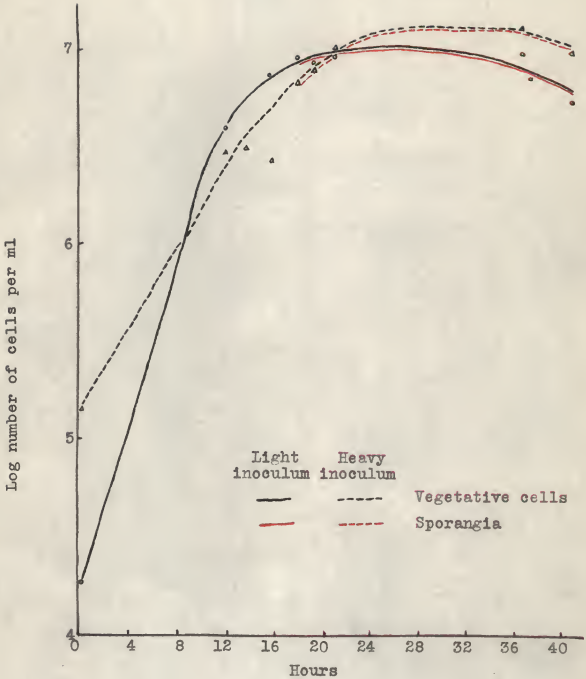

Fig. 5. Bacillus cereus (#1). Growth curve with both light and heavy inocula during later stages of growth.

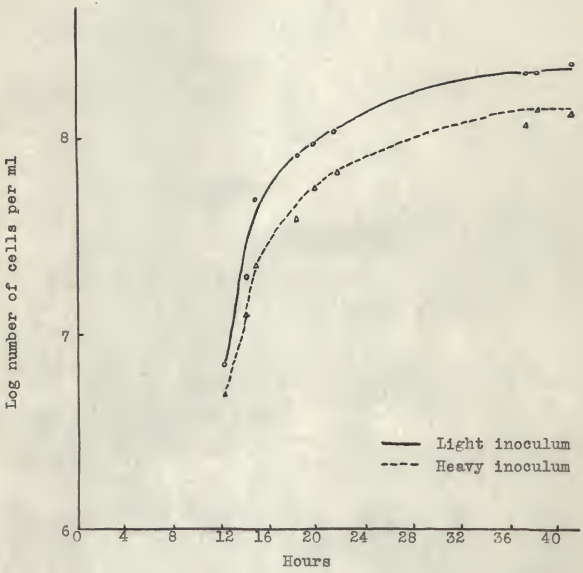

Fig. 6. Bacillus cereus (#1). Growth curve with both light and heavy inocula during later stages of growth, as measured turbidimetrically.

Comparison of Growth and Endospore Formation in Nutrient Broth and in Nutrient Broth Plus One Percent Glucose. The flasks were made up for aeration as previously described except that only 1500 milliliters of nutrient broth were added to each flask. Before autoclaving, one percent glucose was added to the second flask. One milliliter of a spore suspension of Bacillus cereus (#7) was added to each flask.

An average of 10 fields was taken for the direct count, and turbidimetric readings were made after the same time intervals. The majority of samples were taken between 9 and 18 hours. Gray's method of spore staining was used, and duplicate slides were stained with an acid-fast stain during the early period of endospore formation.

. Table 17, Appendix, shows the number of cells per milliliter and the percentage endospore formation of Bacillus cereus (#7) in nutrient broth. The corresponding growth of this organism in nutrient broth plus one percent glucose is shown in Table 18, Appendix. Figure 7 shows the growth curves of this strain of Bacillus cereus in both nutrient broth and in one percent glucose. The turbidimetric readings from both flasks, recorded as millions of cells per milliliter, are shown in Table 19, Appendix, and the corresponding growth curve in Fig. 8.

At 15 hours the cells were vacuolated, and at 16 hours almost all of the cells had proceeded to endospore formation. Growth curves on the nutrient broth and on one percent glucose were almost identical, as was the morphological appearance of

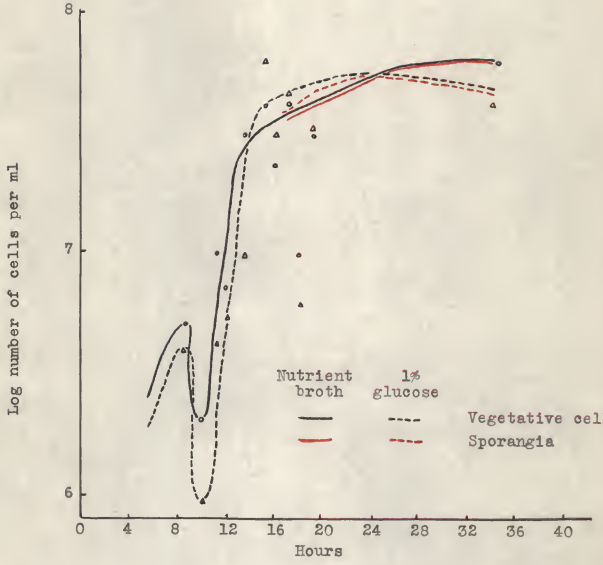

Fig. 7. Bacillus cereus (#7). Growth curve in aerated nutrient broth and in nutrient broth plus one percent glucose.

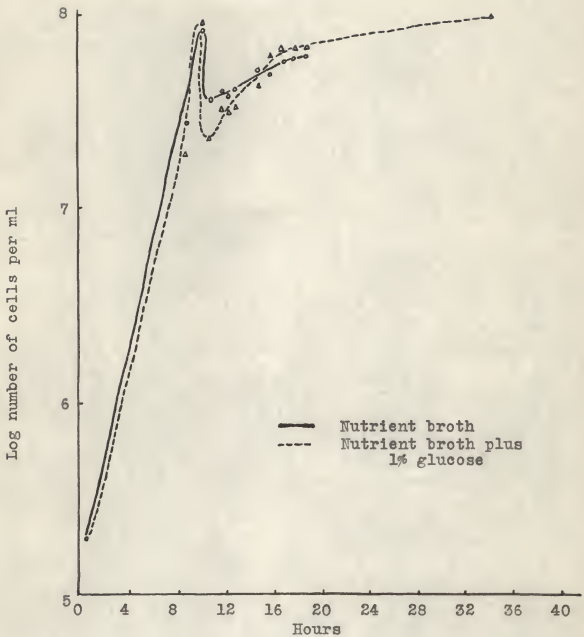

Fig. 8. Bacillus cereus (#7). Growth curve in aerated nutrient broth and in nutrient broth plus one percent glucose, as measured turbidimetrically.

the cells in all stages of growth.

Effect of Varying Agar Concentrations on Growth and Endospore Formation. A series of studies were made on the effect of varying agar concentrations. In the first study, Bacillus cereus (#1) was grown in aerated nutrient broth containing 0.5 percent agar. In the second experiment the same organism was aerated in nutrient broth and in nutrient broth plus 0.2 percent agar. In the third test, Bacillus subtilis (#28) was grown in nutrient broth and in nutrient broth plus 0.2 percent agar. In this experiment direct counts were made from a 1 to 100 dilution and from the undiluted sample.

In the first study, the nutrient broth solution varied from standard nutrient broth in that it contained 0.15 percent Difco bacto-beef extract and 0.5 percent Difco bacto-peptone in addition to 0.5 percent agar. No attempt was made to count cells after 13 hours aeration, as the cells were too highly vacuolated to differentiate between vacuoles and endospores. However, the preparations did show early stages in the growth of this organism. Table 20, Appendix, shows the number of cells per milliliter of samples taken during the early stages of growth, up to 13 hours, and Fig. 9 shows the growth curve of this organism.

At 9½ hours the cells contained faintly perceptible areas of clearing; at 10½ hours the cells were highly vacuolated and a few free endospores were present. At 24 hours the degree of vacuolation reached a maximum and the cells were highly distorted

and swollen. From 24 to 32 hours many free endospores were present, but the unsporulated cells were still highly vacuolated and foamy in appearance.

In the second study, Bacillus cereus (#1) was aerated in standard nutrient broth and in nutrient broth containing 0.2 percent agar. Direct counts were made 20 minutes after aeration was started and at six times between 15 and 21 hours. Turbidimetric readings were made on the nutrient broth control at the same time. Gray's method of spore staining and also an acid-fast stain were used on duplicate slides in the direct count. The number of cells per milliliter and percentage endospore formation are not shown, as endospores were present in 90 percent of the cells at the time the first sample was taken. The amount of growth and the morphological appearance of the cells were identical in the two media. After 15 hours incubation, the nutrient broth flask contained 37 million cells per milliliter, as measured turbidimetrically. The amount of growth increased to 107 million cells per milliliter after 21 hours incubation.

In the third study, Bacillus subtilis (#28) was used in order to compare the amount of growth of this organism with that of Bacillus cereus in nutrient broth and in nutrient broth plus 0.2 percent agar. Three liters of each medium were placed in flasks for aeration. Samples were taken at intervals during a period of 41 hours, and an average of 20 fields was taken for the counts.

In Table 21, Appendix, are shown the number of cells per

milliliter recorded from direct counts of a 1 to 100 dilution and from undiluted samples on aerated nutrient broth. Table 22, Appendix, shows the same counts on aerated nutrient broth plus 0.2 percent agar. The growth curves on both the nutrient broth and nutrient broth plus 0.2 percent agar are shown in Fig. 10, while in Fig. 11 are shown the growth curve made from the direct counts of the 1 to 100 dilution for the same cultures.

No endospore formation occurred in 41 hours with this strain of Bacillus subtilis. In later stages of growth, the cells became banded and stained irregularly. There was little difference in the amount of growth obtained in the nutrient broth and in nutrient broth plus 0.2 percent agar.

DISCUSSION

The preceding aeration experiments with Bacillus cereus show its growth throughout all phases of the growth curve. The early stages of growth, shown in Figs. 1, 2, and 9, show the relatively short lag phase and rapid rate of growth of this organism during the logarithmic growth phase. In all growth curves shown from aerated cultures, the log phase was completed by 13 hours.

Endospore formation commenced during the negative acceleration phase, where cell division was no longer at a maximum, as shown by Figs. 5 and 7. In all cases, the majority of the cells contained endospores before the culture passed over into

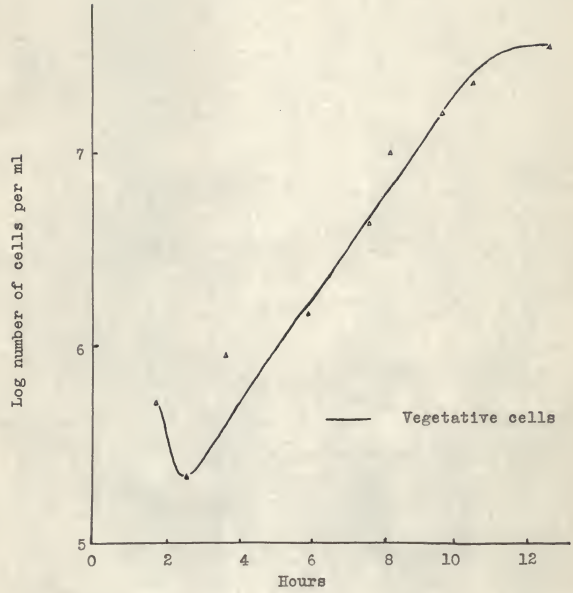

Fig. 9. Bacillus cereus (#1). Growth curve in aerated nutrient broth plus 0.5 percent agar during early stages of growth.

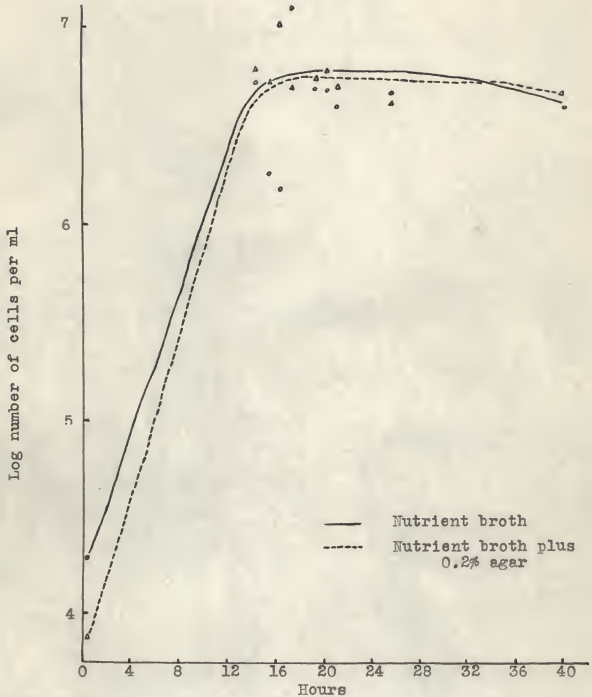

Fig. 10. Bacillus subtilis (#28). Growth curve in aerated nutrient broth and in nutrient broth plus 0.2 percent agar.

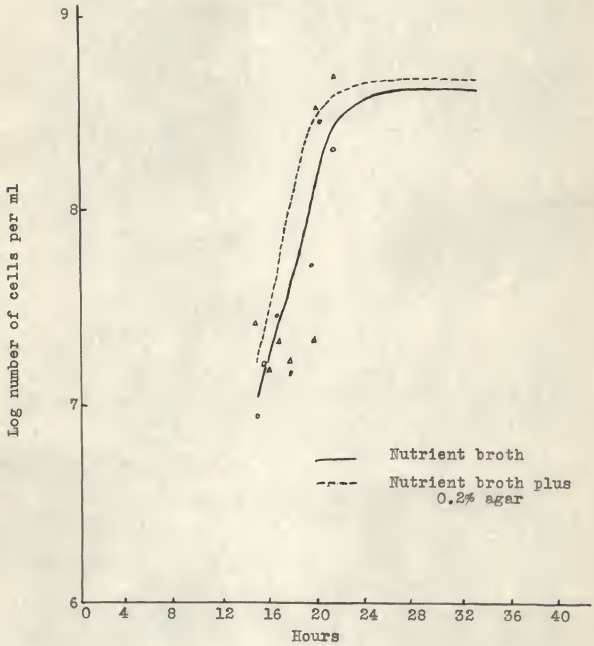

Fig. 11. Bacillus subtilis (#23). Growth curve in aerated nutrient broth and in nutrient broth plus 0.2 percent agar, from direct counts of one to one hundred dilutions of the samples.

the resting phase. The transition was rapid. The cells containing cleared areas had within another hour's incubation formed complete endospores.

The growth curve of Bacillus subtilis (Fig. 10) shows the same relative amount of growth as Bacillus cereus, but no endospore formation occurred; this also was the case in other small-celled species of Bacillus which were aerated. All large-celled species responded to aeration. The failure of Bacillus subtilis and other small-celled species to sporulate may have been due to an inadequate oxygen supply, resulting from the method of aeration used. This does not appear likely, however, as the amount of growth was relatively the same.

Of the factors previously reported as influencing endospore formation none of those tried in these experiments, including agar concentration, added glucose, and varying the amount of the inoculum, caused any change in the time when endospore formation commenced. Also there was little difference in the amount of growth obtained. The effect of these substances on the relative numbers of cells and free endospores at later stages of growth was not determined, but it is possible that these factors might have an effect on the release of endospores from the sporangia.

Endospore formation occurred at a definite time in the growth cycle; i.e. during the negative acceleration phase. Results of this work indicate that factors previously reported as causing changes in endospore formation may be due to changes

in the rate of growth of an organism, rather than to a specific effect on sporulation. A study of factors influencing endospore formation should include more data on the complete growth cycle of the organism. Literature previously reported shows a change in the percentage of free endospores, which may be due to a corresponding increase or decrease in growth of the vegetative cells and may not be a function of endospore formation itself. If the growth cycle of an organism is plotted in terms of the percentage endospore formation at a number of time intervals, then endospore formation is shown as occurring at a definite phase in the growth cycle of the organism. The effect of some added substance upon stimulation or suppression of growth would also be shown.

CONCLUSIONS

With the two strains of Bacillus cereus studied, endospore formation occurred during the negative acceleration growth phase. There was no appreciable difference in growth and endospore formation of Bacillus cereus in aerated nutrient broth and in aerated nutrient broth containing glucose or 0.2 percent agar. A relatively large inoculum also caused no significant change in growth and endospore formation in aerated nutrient broth.

Bacillus subtilis and other small-celled species of the genus Bacillus did not form endospores in response to aeration,

although growth comparable to that of Bacillus cereus was obtained with the strain of Bacillus subtilis studied. This indicates that some factor other than oxygen is involved in the sporulation of Bacillus subtilis.

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APPENDIX

Table 3. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation in unaerated nutrient broth.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
12.25	6.3259	2.12×10^6	0
14.25	6.3220	2.01×10^6	0
16.25	6.2839	1.90×10^6	0
18.25	6.1296	1.35×10^6	0
20.25	6.3709	2.35×10^6	0
22.00	6.3814	2.41×10^6	0
36.25	6.2845	1.43×10^6	0
39.50	6.5422	3.53×10^6	0
42.00	6.0769	1.19×10^6	0
44.50	6.1596	1.44×10^6	0

Table 4. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation in unaerated nutrient broth plus five ml of one and one-half percent colloidal clay.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
12.25	6.4635	2.91×10^6	0
14.25	5.9429	8.77×10^5	0
16.25	6.2088	1.62×10^6	0
18.25	6.5692	3.71×10^6	0
20.25	6.4533	2.84×10^6	0
22.00	6.5470	3.52×10^6	0
36.25	6.3985	2.50×10^6	0
39.50	6.4181	2.62×10^6	0
42.00	6.4149	2.60×10^6	0
44.50	6.5587	3.62×10^6	0
54.25	6.1449	1.39×10^6	0

Table 5. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation in unaerated nutrient broth plus 0.5 percent gelatin.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
12.25	5.9377	8.66×10^5	0
14.25	5.5644	3.66×10^5	0
16.25	5.6995	5.01×10^5	0
18.25	5.9835	4.63×10^5	0
20.25	6.2668	1.85×10^6	0
22.00	6.0088	1.02×10^6	0
36.25	6.6067	4.04×10^5	0
39.50	5.9520	8.95×10^5	0
42.00	5.7317	5.39×10^5	0
44.50	5.9835	9.62×10^5	0

Table 6. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation in unaerated nutrient broth plus 0.5 percent casein.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
12.25	5.9473	8.86×10^5	0
14.25	6.0404	1.10×10^6	0
16.25	5.8666	7.70×10^5	0
18.25	6.0839	1.21×10^6	0
20.25	5.6825	4.81×10^5	0
22.00	-	-	(free spores)

Table 7. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation in unaerated nutrient broth plus 0.06 percent agar.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
12.25	6.2436	1.83×10^6	0
14.25	6.5644	3.66×10^6	0
16.25	6.4777	3.00×10^6	0
18.25	7.0554	1.24×10^7	0
20.25	6.8866	7.70×10^6	0
22.00	6.7028	5.05×10^6	0
36.25	6.4940	3.12×10^6	0
39.25	6.3737	2.37×10^6	0
42.00	5.5644	3.66×10^5	0
44.50	6.5856	3.85×10^6	0

Table 8. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation in unaerated nutrient broth plus 0.3 percent agar.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
12.25	6.6911	4.91×10^6	0
14.25	6.2713	1.87×10^6	0
16.25	6.9946	9.88×10^6	0
18.25	6.8468	7.03×10^6	0
20.25	7.1596	1.44×10^7	0
22.00	6.9791	9.53×10^6	0
36.25	6.7617	5.78×10^6	0
39.50	7.1596	1.44×10^7	0

Table 9. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation in aerated nutrient broth

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
12.25	6.4051	2.54×10^6	0
14.25	7.0852	1.22×10^7	0
16.25	7.0811	1.21×10^7	0
18.25	7.1596	1.45×10^7	0
20.25	-	-	0
22.00	-	-	80
36.25	-	-	95

Table 10. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation in aerated one percent peptone.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
0.92	5.6825	4.81×10^5	0.0
1.83	5.4322	2.71×10^5	0.0
2.83	5.8403	6.93×10^5	0.0
3.59	6.1003	1.26×10^6	0.0
4.08	5.9329	8.57×10^5	0.0
5.08	6.5085	3.23×10^6	0.0
8.59	6.7825	6.06×10^6	0.0
26.08	7.0265	1.06×10^7	93.9
31.42	6.9112	8.15×10^6	93.9
35.42	6.7440	5.55×10^6	93.3

Table 11. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation with the light inoculum during early stages of growth.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
6.00	5.4459	2.79×10^5	0.0
7.58	5.7507	5.63×10^5	0.0
8.58	6.3849	2.43×10^6	0.0
9.25	6.5723	3.73×10^6	0.0
10.00	6.6396	4.36×10^6	0.0
10.50	6.4859	3.06×10^6	0.0
11.00	6.5150	3.26×10^6	0.0
11.50	6.3098	2.04×10^6	0.0
12.00	6.8456	7.01×10^6	0.0
28.25	7.1189	1.31×10^7	98.7

Table 12. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation with the heavy inoculum during early stages of growth.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
6.00	5.7617	5.78×10^5	0.0
7.58	6.3655	2.32×10^6	0.0
8.58	6.6953	4.96×10^6	0.0
9.25	6.0442	1.10×10^6	0.0
10.00	6.3414	2.19×10^6	0.0
10.50	6.5667	3.69×10^6	0.0
11.00	6.9534	8.98×10^6	0.0
11.50	6.5410	3.48×10^6	0.0
12.00	7.0849	1.22×10^7	0.0
28.25	7.1903	1.55×10^7	100.0

Table 13. Bacillus cereus (#1). Number of cells with both light and heavy inocula during early stages of growth, as measured turbidimetrically.

Hours	Millions of cells per ml	
	Light inoculum	Heavy inoculum
6.00	2.5	12.5
7.58	5.0	88.0
8.58	31.0	102.0
9.25	70.0	40.0
10.00	85.0	63.0
10.50	106.0	91.0
11.00	50.0	112.0
11.50	43.0	273.0
12.00	55.0	247.5
28.25	255.0	232.0
31.45	247.5	318.0

Table 14. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation with the light inoculum during later stages of growth.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
0.00	4.2345	1.93×10^4	-
12.25	6.6160	4.13×10^6	0.0
13.92	6.5701	3.72×10^6	0.0
15.33	6.8694	7.58×10^6	0.0
18.00	6.9644	9.21×10^6	97.9
19.58	6.9200	8.32×10^6	99.6
21.00	6.9992	9.99×10^6	99.4
37.08	7.0316	1.08×10^7	99.8
38.33	6.8292	6.75×10^6	93.9
40.83	6.7110	5.14×10^6	99.5

Table 15. Bacillus cereus (#1). Number of vegetative cells and percentage endospore formation with the heavy inoculum during later stages of growth.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
0.00	5.1449	1.40×10^5	-
12.25	6.4818	3.03×10^6	0.0
13.92	6.5313	3.40×10^6	0.0
15.33	6.4587	2.88×10^6	0.0
18.00	6.8516	7.11×10^6	99.7
19.58	6.9026	7.99×10^6	99.5
21.00	7.0022	1.01×10^7	99.9
37.08	7.2021	1.59×10^7	98.5
38.33	7.0821	1.21×10^7	98.7
40.83	7.0353	1.08×10^7	97.6

Table 16. Bacillus cereus (#1). Number of cells with both light and heavy inocula during later stages of growth, as measured turbidimetrically.

Hours	Millions of cells per ml	
	Light inoculum	Heavy inoculum
12.25	7.0	5.0
13.92	20.0	12.5
15.33	49.0	22.5
18.00	84.0	40.0
19.58	96.0	57.0
21.00	110.0	67.0
37.08	225.0	114.0
38.33	225.0	144.0
40.83	247.5	135.0

Table 17. Bacillus cereus (#7). Number of vegetative cells and percentage endospore formation in aerated nutrient broth.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
9.00	6.7363	5.45×10^6	0.0
10.33	6.3159	2.07×10^6	0.0
11.33	7.0092	1.02×10^7	0.0
11.92	6.7667	5.84×10^6	0.0
12.58	6.8292	6.75×10^6	0.0
13.33	7.4359	3.06×10^7	0.0
15.00	7.5963	3.95×10^7	0.0
16.00	7.3637	2.31×10^7	98.0
17.00	7.6170	4.14×10^7	100.0
18.00	6.5856	3.85×10^6	100.0
18.75	7.4749	2.99×10^7	100.0
34.50	7.7828	6.06×10^7	100.0

Table 18. Bacillus cereus (#7). Number of vegetative cells and percentage endospore formation in aerated nutrient broth plus one percent glucose.

Hours	Log cells per ml	Number of cells per ml	Percentage spore formation
9.00	6.6109	4.08×10^6	0.0
10.33	5.9129	8.18×10^5	0.0
11.33	6.6319	4.28×10^6	0.0
11.92	6.7270	5.33×10^6	0.0
12.58	6.7094	5.12×10^6	0.0
13.33	6.9377	8.66×10^6	0.0
15.00	7.7964	6.26×10^7	0.0
16.00	7.4886	3.08×10^7	88.0
17.00	7.6473	4.44×10^7	80.0
18.00	6.7787	6.01×10^6	97.0
18.75	7.5020	3.18×10^7	98.0
34.50	7.6067	4.04×10^7	100.0

Table 19. Bacillus cereus (#7). Number of cells in aerated nutrient broth and in nutrient broth plus one percent glucose, as measured turbidimetrically.

Hours	Millions of cells per ml	
	Nutrient broth	Nutrient broth plus glucose
0.67	2.0	2.0
9.00	178.0	186.0
10.33	860.0	890.0
11.33	350.0	225.0
11.92	390.0	320.0
12.58	375.0	320.0
13.33	410.0	360.0
15.00	485.0	430.0
16.00	485.0	615.0
17.00	550.0	665.0
18.00	600.0	630.0
19.75	600.0	635.0
34.50	-	930.0

Table 20. Bacillus cereus (#1). Number of vegetative cells in aerated nutrient broth plus 0.5 percent agar during early stages of growth.

Hours	Log cells per ml	Number of cells per ml
1.67	5.7469	5.58×10^5
2.42	5.3259	2.12×10^5
3.42	5.9612	9.15×10^5
5.59	6.1710	1.48×10^6
6.50	6.1632	1.47×10^6
7.59	6.5150	3.27×10^6
8.25	7.0472	1.12×10^7
9.59	7.1844	1.54×10^7
10.59	7.2230	1.67×10^7
12.59	7.5633	3.67×10^7

Table 21. Bacillus subtilis (#28). Number of cells in aerated nutrient broth.

Hours	Undiluted sample		1:100 dilution of sample	
	Log cells per ml	Number of cells per ml	Log cells per ml	Number of cells per ml
0.20	4.2645	1.93×10^4	-	-
15.00	6.7362	5.45×10^6	6.9377	8.66×10^6
16.00	6.2448	1.76×10^6	7.2010	1.58×10^7
17.00	6.1915	1.55×10^6	7.4633	2.91×10^7
18.00	6.7102	5.13×10^6	7.2010	1.58×10^7
19.75	6.8936	7.83×10^6	7.3814	2.41×10^7
20.75	6.6647	4.62×10^6	8.4149	2.60×10^8
21.75	6.5817	3.82×10^6	8.2735	1.88×10^8
26.50	6.6472	4.44×10^6	-	-

Table 22. Bacillus subtilis (#28). Number of cells in aerated nutrient broth plus 0.2 percent agar.

Hours	Undiluted sample		1:100 dilution of sample	
	Log cells per ml	Number of cells per ml	Log cells per ml	Number of cells per ml
00.20	3.8814	7.61×10^3	-	-
15.00	6.7924	6.20×10^6	7.4067	2.55×10^7
16.00	6.7332	5.41×10^6	7.2010	1.58×10^7
17.00	7.0155	1.04×10^7	7.3057	2.02×10^7
18.00	6.6528	4.50×10^6	7.2139	1.64×10^7
19.75	6.7094	5.12×10^6	7.3057	2.02×10^7
20.75	6.7536	5.67×10^6	8.5150	3.27×10^8
21.75	6.6697	4.67×10^6	8.6510	4.48×10^8
26.50	6.5892	3.88×10^6	-	-