Effect of pH on Sporulation of *Bacillus stearothermophilus*

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An improved broth medium was developed for high growth yields of *Bacillus subtilis* var. *niger* NCIB 8649, *Bacillus cereus* NCIB 9373, and *Bacillus stearothermophilus* NCIB 8919 and ATCC 7953. Sporulation was abundant (1.1 × 10^8 *B. subtilis* var. *niger* and 9.2 × 10^7 *B. cereus* per ml) at an initial pH of 7.0. Sporulation of both strains of *B. stearothermophilus* took place (1.9 × 10^7 and 2.4 × 10^7/ml, respectively) in this medium when initial pH values of 7.7 to 8.7 were used.

*Bacillus stearothermophilus* sporulates poorly in most liquid bacteriological media; attempts have been made, therefore, to develop media in which a significant number of spores are produced, but little work has been done on the environmental and pH aspects of sporulation. Several workers (3, 5, 7, 8, 11) developed special media in which *B. stearothermophilus* sporulates, yet the degree of sporulation obtained in this laboratory, even with these special media, was not consistently high and abundant.

In the experiments reported here, a reproducible method for obtaining high yields of spores of *B. stearothermophilus* in an improved broth medium suggested by Thompson and Thames (11) at pH 7.7 to 8.7 is described. For comparison, changes in the pH of the sporulation medium and the degree of sporulation of *Bacillus cereus* and *Bacillus subtilis* var. *niger* were studied.

MATERIALS AND METHODS

Microorganisms and inocula. *B. stearothermophilus* ATCC 7953, *B. stearothermophilus* NCIB 8919, *B. cereus* NCIB 9373, and *B. subtilis* var. *niger* NCIB 8649 were used as the test organisms. The two strains of *B. stearothermophilus* are widely used in thermal death time and inoculated-pack studies (2, 11). Freeze-dried cultures were streaked on nutrient agar (Difco) slants, incubated for 12 h at the required temperatures (60°C for *B. stearothermophilus* ATCC [11], 37°C for *B. subtilis* var. *niger* and *B. cereus*, and 56°C for *B. stearothermophilus* NCIB [2]), and stored at 4°C.

Inocula were prepared by washing the growth from an appropriate culture into 100 ml of the medium described by Thompson and Thames (11). After 12 h of incubation, cells were removed by centrifugation and used for inoculation of the sporulation media.

Sporulation media. Ten milliliters of inoculum with an optical density of 0.70 at 580 nm was added to a 250-ml flask containing 150 ml of sporulation medium. The sporulation medium consisted of: peptone (Oxoid), 0.30%; tryptone (Difco), 0.25%; yeast extract (Difco), 0.40%; beef extract (Difco), 0.25%; K₂HPO₄, 0.20%; and MnSO₄, 10 μg/ml. The pH was adjusted from 5.0 to 9.50 with 0.1 N NaOH or 0.1 N H₃PO₄, and the media were autoclaved (121°C) for 20 min. After sterilization the pH ranged from 5.20 to 9.50. The addition of inocula did not change the pH of the medium. In modifying the medium suggested by Thompson and Thames (11) various components were omitted or added, one at a time, to determine their effect on sporulation of these strains of *B. stearothermophilus*. The components were also tested in different concentrations and at different initial pH values to determine the optimal concentrations and pH values stimulatory to sporulation. In the final selection of the concentration of the individual compounds of the medium, the choice was usually made to favor increased sporulation in *B. stearothermophilus* 7953, which normally produced the lower number of spores; a solid medium with the above composition at pH 8.0 gave a good crop of spores, which was not counted.

Measurements. A PYE (PYE Unicam Ltd., Cambridge, England) pH meter equipped with a digital recorder was used to automatically record the pH of the growing culture at 20-min intervals. Plating and microscopic examinations were used to estimate sporulation and compare viable with total numbers. For plating, 25-ml samples of each culture were transferred to stoppered flasks and heated to 110°C for 5 min after temperature equilibration (5 min) for *B. stearothermophilus* (11), or to 78 to 80°C for 20 min for *B. cereus* and *B. subtilis* var. *niger* (4), to destroy vegetative cells. Thus, only spores were determined by outgrowth in triplicate on tryptone-glucose extract agar. Dilutions were made in distilled water and viable counts were made after incubation for 48 h. The percentage of sporulation of an organism has been determined on the basis of the number of colonies obtained before and after a given heat treatment; however, this method may be inaccurate for certain sporeforming organisms, because in some instances there is a heat activation requirement for
spores before they will undergo optimal germination and outgrowth. The total colony counts of both species of *B. stearothermophilus* spores were the same before and after heat treatment at 110°C for 5 min. This suggested that the heat treatment did not stimulate additional germination and outgrowth of the spores. For this reason, the results presented here are reported as the percentage of total cells. A second reason for this procedure was that the low number of spores produced by these strains in some media made it impossible to accurately measure the total sporulation by direct microscopic counts. Microscopic estimations of sporulation in wet mounts were made by use of phase-contrast microscopy; the percentage of sporulation was calculated on the basis of 250 organisms.

**RESULTS**

Changes in pH of the sporulation medium with an initial pH 7.0 ± 0.1 for four species of bacilli and the points at which sporulation started are presented in Fig. 1. After a lag period, nearly 3.5 h for all four species, a sudden fall in pH of the medium was observed, followed by a sharp rise in pH of the medium in cultures of *B. subtilis* var. *niger* and *B. cereus*. Observed changes in pH for *B. cereus* and *B. subtilis* var. *niger* are typical of *Bacillus* spp. cultured in complex media (6, 7, 10). The presence of spores was detected in these two species, but in *B. stearothermophilus* the pH did not rise and few spores were detected.

Changes in the pH of the medium with an initial pH of 8.5 for both strains of *B. stearothermophilus* and the points at which sporulation started are presented in Fig. 2. The patterns of changes in pH were similar to the other *Bacillus* spp., but the pH was never less than 6.8 in this medium. The percentage of sporulation of two *B. stearothermophilus* strains in media of different initial pH values is plotted in Fig. 3. Below pH 5.0 and above pH 9.0 the growth of both strains of *B. stearothermophilus* was suppressed. Between these two values there was little difference in the amount of growth (vegetative cells plus spores) after 16 h of incubation. Maximum sporulation (about 88%) occurred between pH 7.7 to 8.7. Aeration by shaking did not increase the amount of growth and had no effect on the percentage of sporulation.

**DISCUSSION**

It has been shown (6, 10) that during the exponential period of growth of *B. cereus* T pyruvic and acetic acids are released, bringing about an initial fall in the pH of the culture. When sporulation began, the acids were utilized and the pH rose. The medium used in the present work did not contain added glucose; however, it is possible that some of the ingredients (particularly yeast extract) contained car-
bohydrates from which acids were formed. The pattern of events described for *B. cereus* T (6, 10) would explain the cause of the changes in the pH of the medium which occurred in the present investigation.

When *B. stearothermophilus* ATCC 7953 and *B. stearothermophilus* NCIB 8919 were cultured in the medium at an initial pH of 7.0 there was a decrease in the pH of the culture to about 5.5, after which the pH remained unchanged; few spores were produced. When grown in a medium that initially was adjusted to pH 7.7 to 8.7, however, the pattern of changes in pH of the culture was similar to those observed with the other species of bacilli, and sporulation of the *B. stearothermophilus* occurred. This indicates that the enzymes of *B. stearothermophilus* which are responsible for utilizing organic acids during spore production may not be induced or may be inactive at low pH values produced during growth of vegetative cells, and, therefore, sporulation does not occur. It may be that at pH 7.7 to 8.7 these enzymes are induced, become activated, or are not inactivated, and sporulation occurs.

Alternatively, it is possible that the release and accumulation of cell wall lytic enzymes or antibiotics (1, 9) produced during growth and at the beginning of sporulation are more active at low pH, causing damage to the remaining vegetative cells before sporulation begins.

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**LITERATURE CITED**