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Effect of Soybean Casein Digest Agar Lot on Number of *Bacillus stearothermophilus* Spores Recovered†

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In recent years it has become increasingly apparent that *Bacillus stearothermophilus* spores are affected by various environmental factors that influence the performance of the spores as biological indicators. One environmental factor is the recovery medium. The effect of different lots of commercial soybean casein digest agar on the number of colony-forming units per plate was examined in two series of experiments: (i) several lots of medium from two manufacturers were compared in single experiments, and (ii) paired media experiments with four lots of medium were carried out and yielded three-point survivor curves. The results demonstrate that commercial soybean casein digest agar is variable on a lot-to-lot basis. The variation was lowest when recovering unheated or minimally heated spores and increased greatly with the severity of heating.

The spores of *Bacillus stearothermophilus* are widely used as biological indicators in the food, drug, and health care industries to design, validate, or monitor steam sterilization processes. Our laboratory has been investigating the applications, advantages, and limitations of bacterial spores as biological monitors, and we have become increasingly aware of the effect of environmental factors on the number of recoverable spores. One of the most important of the environmental variables is the recovery medium.

Several investigators (2, 3, 5) have found that different formulations of recovery media produce various levels of viability for heated spores. However, there are no reports of the effect of different lots of the same medium formulation.

The purpose of this investigation was to determine whether differences existed in the *B. stearothermophilus* spore recovery capabilities of different lots of commercial soybean casein digest agar from two manufacturers when plate count procedures were used.

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MATERIALS AND METHODS

Two series (I and II) of experiments were carried out.

Experimental plan. (i) Series I. Several lots of medium from two manufacturers were evaluated in each of four experiments. In the first two tests, three lots of medium from manufacturer B (B-1, B-2, and B-3) and three lots from manufacturer D (D-1, D-2, and D-3) were evaluated. In the third and fourth experiments, media B-1, B-2, B-3, D-1, and D-2 were tested.

(ii) Series II. Five experiments were carried out, each evaluating two lots of medium. Medium B-4 was used as a standard and was evaluated in each test along with a second lot of medium (B-5, B-6, D-4, or D-5).

Spores. In series I experiments two strains of *B. stearothermophilus* were used: (i) one spore preparation was produced from strain FS1518, received from C. F. Schmidt, Continental Can Co., Chicago, Ill., and (ii) two spore preparations were produced from strain ATCC 7953.

Series II experiments utilized one spore crop grown from strain ATCC 7953.

All spore crops were grown at 55°C for 48 h on nutrient agar supplemented with 5 μg of $MnSO_4$ per liter. The spores were cleaned by using insonation to free them from vegetative debris and repeatedly washed with distilled water by using centrifugation. After cleaning, the spores were resuspended in 50× Butterfield buffer (1).

Spore carriers. Plastic rod biological indicator units (BIUs) similar to those described by Pflug et al. (6) were used as spore carriers. Each BIU was filled with 0.28 ml of spore suspension (5×10^6 spores) and was stored at 4°C until tested.

Heating procedures. For each experiment three replicate BIUs were heated at 121.0°C at each of several heating times with a miniature retort. After heating, the BIUs were cooled and held in an ice water bath until spore recovery procedures were started. In series I experiments three unheated BIUs were also evaluated.

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Recovery procedures. A 0.1-ml sample of the spore suspension from each BIU was placed in 100 ml of Butterfield dilution buffer. After shaking, duplicate 0.01-, 0.1-, or 1.0-ml samples of the dilution were plated for each medium lot to be evaluated. Thirty milliliters of the appropriate medium lot was poured into each plate. The plates were incubated at 55°C for 48 h, and the number of colony-forming units was counted.

RESULTS

Series I, statistical analysis of the data. Assessing the efficacy of different medium lots for spore recovery was measured by the number of colony-forming units per plate. The data for each experiment were treated as split-plot designs with the whole plot in a completely randomized design. A whole plot was taken to be the number of colony-forming units for a single BIU. A logarithmic transformation of the data was made and used in the analysis because we thought that the coefficient of variation was likely to be constant, and effects were more likely to be proportional than additive. Duplicate plate counts were handled as subsamples. This is generally the more conservative approach and has the added advantage of being equivalent to doing the analysis on the means of the logarithm of the two plate counts, which should give a better approximation to normal errors.

The analysis of variance is presented in Table 1. The F tests for split-plot treatments and interaction between medium lot and heating time were significant at the 99% level in all four experiments.

The difference between the media of manufacturers D and B changed significantly between zero heating time and the greatest heating time in all four experiments. In all cases the media of manufacturer D produced higher average counts of heated spores than the media of manufacturer B. The significance level of these results was in excess of 99% for three experiments and was 98% for the fourth experiment.

The logarithmic means for the four experiments are shown in Table 2. Bonferroni's inequality (4) was used to test, at each time at the 95% levels, the differences of the media of the two manufacturers. We found no significant difference at time zero in three of the four experiments. The treatment means for medium B-2 were smaller than those of B-1 and B-3 for all heating times in all four experiments. To investigate the possibility that the difference in manufacturers was simply due to the low counts for medium B-2, in Table 2 we also compared the mean of B-1 and B-3 with the mean of the media from manufacturer D at each time. Significant differences still existed between manufacturers when medium B-2 was eliminated.

TABLE 1. *Series I: analysis of variance table*[a]

Expt	Source[b]	df	Sum of squares	Mean square
1	HT	3	16.589	5.5298
	Error, w	8	4.6712	0.5839
	M	5	2.8561	0.57121
	M × HT	15	1.1016	0.073437
	Error, sp	40	0.17870	0.0044675
	Error, sub	72	0.30917	0.0042940
	Total	143	25.706	
	GM	1	628.64	
2	HT	2	2.4680	1.2340
	Error, w	6	0.032626	0.0054376
	M	5	0.50353	0.10071
	M × HT	10	0.68230	0.068230
	Error, sp	30	0.12031	0.0040105
	Error, sub	54	0.13995	0.0025917
	Total	107	3.9467	
	GM	1	352.64	
3	HT	3	16.178	5.3925
	Error, w	8	0.031017	0.0038771
	M	4	1.1771	0.29429
	M × HT	12	0.51172	0.042644
	Error, sp	32	0.054033	0.0016885
	Error, sub	60	0.15370	0.0025617
	Total	119	18.105	
	GM	1	553.69	
4	HT	2	1.2143	0.60717
	Error, w	6	0.048101	0.0080169
	M	4	0.81271	0.20318
	M × HT	8	0.39252	0.049065
	Error, sp	24	0.22404	0.0093350
	Error, sub	45	0.44206	0.0098234
	Total	89	3.1338	
	GM	1	209.68	

[a] F tests for split plot treatments and interaction are significant at 99% level in all four experiments.

[b] Abbreviations: HT, heating time; w, whole; M, media; sp, split; sub, subsamples; GM, grand mean.

The plate count data were analyzed to determine whether the relationship between pairs of medium lots changed as the heating time increased. The Bonferroni method was used to keep these tests collectively at about the 95% level.

For manufacturer B, experiments 1 and 2, media B-1 and B-3 produced equivalent counts at all heating times. Medium B-2 gave lower counts than B-1 and B-3 at the last two heating times. In experiments 3 and 4, medium B-2

TABLE 2. *Series I: logarithmic mean of plate counts and statistical significance of data using Bonferroni's inequality*

Expt	Heating time (min)	Log means of plate counts[a] on medium:						Mean B − mean D[b]	Significance[c]	(Mean B-1 + B-3) − (mean D)[d]	Significance[c]
		B-1	B-2	B-3	D-1	D-2	D-3				
1	0	2.336	2.403	2.299	2.406	2.324	2.247	0.02033	N.S.	−0.0082	N.S.
	4.8	1.952	1.929	1.973	2.093	2.030	1.692	0.01300	N.S.	0.0242	N.S.
	9.8	2.523	2.368	2.531	2.670	2.587	2.146	0.00833	N.S.	0.059	N.S.
	14.8	1.647	1.313	1.697	1.911	1.827	1.241	−0.10733	Sig.	0.012	N.S.
2	0	1.774	1.891	1.730	1.829	1.849	1.727	−0.00333	N.S.	−0.050	N.S.
	3.8	1.639	1.418	1.631	1.717	1.681	1.666	−0.12533	Sig.	−0.053	N.S.
	7.8	1.949	1.733	2.054	2.185	1.968	2.083	−0.16666	Sig.	−0.0792	Sig.
3	0	2.653	2.648	2.595	2.704	2.677		−0.0585	Sig.	−0.0685	Sig.
	3.8	2.263	2.162	2.281	2.375	2.325		−0.1147	Sig.	−0.078	Sig.
	5.8	1.970	1.772	2.027	2.190	2.039		−0.1905	Sig.	−0.116	Sig.
	7.8	1.559	1.375	1.745	1.901	1.700		−0.2408	Sig.	−0.1485	Sig.
4	0	1.558	1.489	1.447	1.573	1.593		−0.085	N.S.	−0.0805	N.S.
	3.8	1.673	1.500	1.699	1.793	1.665		−0.105	Sig.	−0.043	N.S.
	5.8	1.324	1.087	1.462	1.578	1.456		−0.226	Sig.	−0.124	Sig.

[a] Logarithmic mean of six plate counts: duplicate plate counts for three replicate BIUs.
[b] Logarithmic mean of media B-1, B-2, and B-3 minus logarithmic mean of media D-1, D-2, and D-3 or D-1 and D-2.
[c] Statistical significance of differences at 95% level using Bonferroni's inequality: N.S., not significant; Sig., significant.
[d] Logarithmic mean of media B-1 and B-3 minus logarithmic mean of media D-1, D-2, and D-3 or D-1 and D-2.

produced lower counts than B-1 and B-3 at all heating times. Medium B-1 counts were smaller than B-3 at the longest heating time.

For manufacturer D, there were two experiments in which D-1, D-2, and D-3 were compared and four experiments in which D-1 and D-2 were compared. In experiment 1 there was no significant difference between D-1 and D-2 at all heating times. Medium D-3 gave lower counts than D-1 and D-2 at all heating times. For experiment 2, D-1 and D-3 were equivalent at all heating times, and D-2 produced lower counts at only the longest heating time. For experiment 3, D-2 produced lower counts than D-1 at the two longest heating times, but in experiment 4, these two media were equal.

Series II experiments. The results of series I experiments caused our laboratory to change media purchasing procedures. A quantity of at least 25 lb (ca. 11.3 kg) of one lot of soybean casein digest agar is ordered from a local distributor, with the understanding that 1 lb of the lot can be evaluated for effectiveness before the remaining 24 lb are accepted.

The evaluation test is carried out on a two-media basis. The existing lot that is satisfactory is used as a staridard and is tested with the new lot. Series II experiments are the results of such evaluations. Medium B-4 was the standard lot and was used in every test along with the new medium lot (B-5, B-6, D-4, or D-5).

The mean numbers of colony-forming units per plate for the five experiments are presented in Table 3.

TABLE 3. *Series II: paired media experiments for B. stearothermophilus spores heated at 121°C*

Expt	Heating time (min)	Mean plate count[a] (medium)	
5[b]	2	323.3 (B-4)	324.7 (B-5)
	8	36.2 (B-4)	22.8 (B-5)
	16	318.2 (B-4)	96.7 (B-5)
6[b]	2	318.0 (B-4)	327.3 (B-5)
	8	42.0 (B-4)	32.5 (B-5)
	16	254.5 (B-4)	93.7 (B-5)
7	2	277.2 (B-4)	326.3 (D-4)
	8	34.5 (B-4)	71.5 (D-4)
	16	19.3 (B-4)	106.7 (D-4)
8	2	284.5 (B-4)	261.3 (D-4)
	7	62.3 (B-4)	37.7 (D-4)
	16	36.7 (B-4)	6.7 (D-4)
9	2	242.8 (B-4)	290.8 (D-5)
	7	69.7 (B-4)	87.7 (D-5)
	16	28.5 (B-4)	53.8 (D-5)

[a] Duplicate plate counts for three replicate BIUs, mean of six plate counts.
[b] Replicate experiments.

As in series I experiments, media from manufacturer D produced the highest plate counts, and the effect of the different lots on the number of colony-forming units per plate increased with increasing heating time.

Using representative data from these experiments, three-point survivor curves were plotted and point-to-point D(121)-values were calculated (Fig. 1).

The effect of different medium lots on a survivor curve is very apparent in Fig. 1. The number of surviving spores after 16 min of heating was 1 order of magnitude larger with medium D-5, which produced the largest number of survivors, than with medium B-5 or B-6, which gave the smallest number of survivors.

DISCUSSION

The formula, final pH, and method of preparation of the media were the same for both manufacturers. Literature supplied by manufacturer B indicated that the quality of each medium lot was biologically evaluated by using nonsporeforming, nutritionally fastidious microorganisms. These facts tend to instill a false sense of assurance in the equality of different

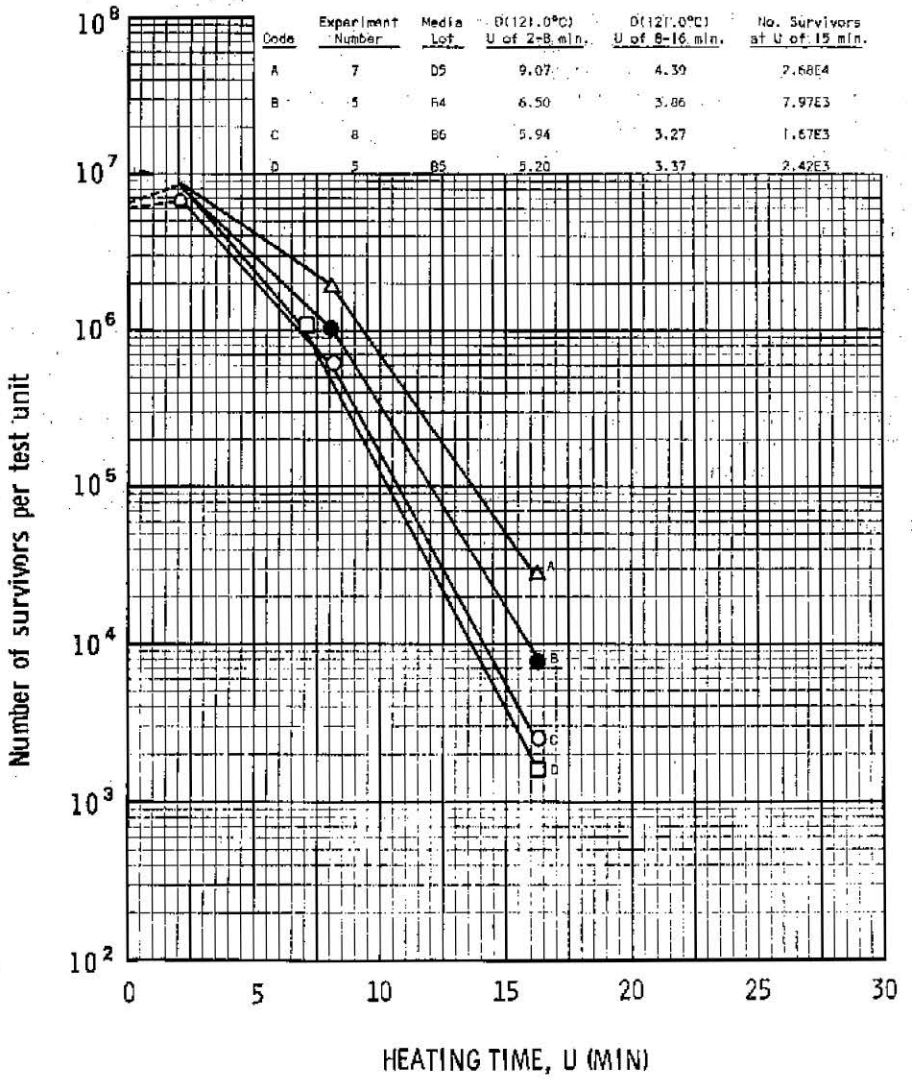

FIG. 1. *Survivor curves for Bacillus stearothermophilus spores heated at 121.0°C in plastic rod BIUs recovered by using four different lots of soybean casein digest agar.*

lots of medium from the same or different manufacturers.

Soybean casein digest agar contains 1.5% of a papaic digest of soybean meal, 0.5% of a pancreatic digest of casein, 0.5% sodium chloride, and 1.5% agar. Specifications for the above ingredients, which are obtained from organic sources, are listed in *The United States Pharmacopeia XIX* (7), and both manufacturers state that their ingredients comply with these specifications. However, the nutritional value of any medium component derived from biological material will probably vary greatly on a lot-to-lot basis. The complexity of the composition of the biologically derived ingredients is such that complete analyses and quality control by the manufacturer would be virtually impossible and would make the cost of the medium prohibitive.

The results of these experiments indicate that the plate count results for heated *B. stearothermophilus* spores may vary with the manufacturer and lot of soybean casein digest agar used. Experimenters can expect to find differences in the media from different manufacturers, and variability among lots of media from the same manufacturer may be as great as from different manufacturers.

Curran and Evans (3) and Cook and Gilbert (2) reported that differences in the number of spores recovered by using different medium formulas increased with heat stress, indicating that heated spores have an increased sensitivity to the nutritional environment. Our results with different lots of medium of the same formulation confirm this conclusion.

For microbiologists doing heat destruction studies on *B. stearothermophilus* spores the message is clear: the performance of the recovery medium must be validated using heated spores when media from two different manufacturers are used. The critical performance test is with heated *B. stearothermophilus* spores.

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LITERATURE CITED

1. **Butterfield, C. T.** 1933. The selection of dilution waters for bacteriological examination. Public Health Rep. **48**: 681–691.
2. **Cook, A. M., and R. J. Gilbert.** 1968. Factors affecting the heat resistance of *Bacillus stearothermophilus* spores. I. The effect of recovery conditions on colony count of unheated and heated spores. J. Food Technol. **3**:285–293.
3. **Curran, H. R., and F. R. Evans.** 1937. The importance of enrichment in the cultivation of bacterial spores previously exposed to lethal agencies. J. Bacteriol. **34**: 179–189.
4. **Dunn, O. J.** 1961. Multiple comparisons among means. J. Am. Stat. Assoc. **56**:52–64.
5. **Pflug, I. J., M. Scheyer, G. M. Smith, and M. Kopelman.** 1979. Evaluation of recovery media for heated *Clostridium sporogenes* spores. J. Food Protect. **42**: 946–948.
6. **Pflug, I. J., G. Smith, R. Holcomb, and R. Blanchett.** 1980. Measuring sterilizing values in containers of food using thermocouples and biological indicators. J. Food Protect. **43**:119–123.
7. **The United States Pharmacopeia XIX.** 1975. Mack Publishing Co., Easton, Pa.