APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1977, p. 377-381

Vol. 34, No. 4
Printed in U.S.A.

Recovery of Spores of *Clostridium botulinum* in Yeast Extract Agar and Pork Infusion Agar After Heat Treatment†

THERON E. ODLAUG* AND IRVING J. PFLUG

Department of Food Science and Nutrition, School of Public Health, University of Minnesota, Minneapolis, Minnesota 55455

Received for publication 18 April 1977

Yeast extract agar, pork infusion agar, and modifications of these media were used to recover heated *Clostridium botulinum* spores. The *D*- and *z*-values were determined. Two type A strains and one type B strain of *C. botulinum* were studied. In all cases the *D*-values were largest when the spores were recovered in yeast extract agar, compared to the *D*-values for spores recovered in pork infusion agar. The *z*-values for strains 62A and A16037 were largest when the spores were recovered in pork infusion agar. The addition of sodium bicarbonate and sodium thioglycolate to pork infusion agar resulted in *D*-values for *C. botulinum* 62A spores similar to those for the same spores recovered in yeast extract agar. The results suggest that sodium bicarbonate and sodium thioglycolate should be added to recovery media for heated *C. botulinum* spores to obtain maximum plate counts.

There is no standard medium used for recovery of *Clostridium botulinum* type A and B spores in wet-heat thermal resistance studies. The different types of recovery media that have been used for *C. botulinum* spores include fresh-meat infusions such as beef heart (4), beef (15), pork (12, 17), pork-pea (9), and liver (14) and formulated media such as yeast extract (8), tryptone-yeast extract (6), and T-Best agar (1).

In survivor curve or fraction-negative studies (10), if a medium is used where some viable spores do not germinate and grow to produce colonies, then the calculated D_T-value will be underestimated. The objective of this study was to compare the effectiveness of pork infusion agar (12), yeast extract agar (3, 16), and modifications of these media for recovery of *C. botulinum* spores in survivor curve thermal resistance studies and to determine how the recovery media affect the calculated D_T- and *z*-values.

(Part of this work is from a thesis submitted by T.E.O. to the Graduate School, University of Minnesota, in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Spores. The source of the strains of *C. botulinum* used in this study and preparation of the spore suspensions were previously described (8).

Yeast extract agar (3). Yeast extract agar contained: yeast extract (BBL), 10.0 g; soluble starch (BBL), 1.0 g; K_2HPO_4, 2.0 g; agar, 15 g; distilled water, 1,000 ml; pH 7.2. The medium was sterilized for 15 min at 121°C in 500-ml amounts in 1,000-ml Erlenmeyer flasks. Immediately before the plates were poured, the following additions were aseptically made to each flask (500 ml): 6.25 ml of a 10% sodium thioglycolate solution, 6.25 ml of a 40% dextrose solution, and 12.5 ml of a 4% sodium bicarbonate solution. Dextrose was sterilized by filtration through a 0.45-μm membrane filter, and the other two compounds were sterilized by autoclaving for 15 min at 121°C.

Pork infusion agar (11). One pound of fresh pork from which the fat had been removed was ground and mixed with 1,000 ml of distilled water, and the mixture was boiled for 1 h. The pork particles were removed by filtering through two layers of cheesecloth, between which was a pad of glass wool. The filtrate was cooled in a refrigerator; after cooling, the fat was skimmed off, and the volume was made up to 1 liter with distilled water. The broth was placed in a straight-walled beaker, and the following ingredients were added: peptone, 5 g; tryptone, 1.5 g; K_2HPO_4, 1.25 g; soluble starch, 1 g; dextrose, 1 g.

The medium was heated and stirred to dissolve these ingredients. The pH was adjusted with 1.0 N NaOH to 7.6. Fifteen grams of agar was added, and the medium was autoclaved for 25 min at 110°C to liquefy the agar and promote formation of a precipitate. While still hot, the medium was placed in a refrigerator at about 4°C until it solidified. The solidified medium was then removed from the beaker by loosening it from the wall with a spatula and inverting the beaker. The layer containing any settled precipitate was trimmed off and discarded. The remaining agar was cut into small cubes, which

† Scientific Journal Series no. 9897, Minnesota Experiment Station, St. Paul, MN 55108.

were returned to the beaker, and the medium was reliquefied by autoclaving for 25 min at 110°C. It was then dispensed into tubes in 20-ml amounts and sterilized by autoclaving for 30 min at 121°C.

Modified pork infusion agar. In some tests the pork infusion agar was modified by adding 0.25 ml of a 10% sodium thioglycolate solution and 0.5 ml of a 4% sodium bicarbonate solution to each tube of 20 ml of medium immediately before pouring a plate. This medium is identified as pork infusion BT agar.

Heating substrate. Sorensen's 0.067 M phosphate buffer (pH 7.0) was prepared by mixing 61.1 ml of a stock solution of 0.067 M disodium phosphate (Na_2HPO_4) with 38.9 ml of a stock solution of 0.067 M monopotassium phosphate (KH_2PO_4). The prepared buffer was autoclaved for 15 min at 121°C.

Heating unit and heating procedure. The aluminum thermal death time unit-miniature retort system (8) was used in this study. The procedure for inoculating and heating the units was previously described by Odlaug and Pflug (8).

Recovery of heated spores. Samples of the heated substrate containing the spores were either plated directly in duplicate or were added to dilution blanks and plated in duplicate. After the recovery medium (pork infusion agar or yeast extract agar) was added and the agar had solidified, the plates were inverted. The plates were then placed in an anaerobic jar under a GasPak hydrogen-carbon dioxide atmosphere and incubated at 32°C for 9 days.

Some experiments were carried out where yeast extract agar and pork infusion agar were used in parallel tests. In these tests a portion of each sample for every dilution was deposited in four plates, two plates for each of the two media. Four additional experiments were carried out where yeast extract agar, modified yeast extract agar (no bicarbonate and thioglycolate added), pork infusion agar, and pork infusion BT agar were used in parallel tests. In these tests a portion of each sample for every dilution was deposited in eight plates, two plates for each of the four media.

Analysis of colony count data. The semilogarithmic model was used as the basis for correlating the heat destruction data and determination of D-values (time for a 90% reduction in the microbial population) (8). In the analysis procedure the survivor data for the unheated controls (N_0) were separated from the survivor data at the several heating times. The survivor data for the several test heating times (not including the unheated controls) were correlated by using the simple linear regression of the logarithm of the survivors versus heating time. The slope of the regression line was used to estimate the thermal resistance parameter (D). The zero-time intercept of the regression line (Y_0) was calculated. The Y_0 and N_0 values were used to calculate the intercept ratio; intercept ratio = $\log Y_0/\log N_0$.

RESULTS

The results of the survivor curve tests for *C. botulinum* spores heated in Sorensen's 0.067 M phosphate buffer (pH 7) are summarized in Table 1. Two *C. botulinum* type A strains and one type B strain were tested. All three strains showed larger $D_{\frac{1}{4}}$-values when yeast extract agar was used as the recovery medium.

The z-values for the type A spores are also listed in Table 1. For strains 62A and A16037 the z-values for spores recovered using pork infusion agar were larger than those for spores recovered using yeast extract agar.

Using the 62A spores, a number of parallel tests were carried out comparing yeast extract agar, pork infusion agar, pork infusion BT agar (thioglycolate and bicarbonate added), and modified yeast extract agar (no thioglycolate or bicarbonate added). The results of these tests are summarized in Table 2. The results indicate that the D-values for *C. botulinum* 62A spores recovered in pork infusion BT agar were similar to those obtained for spores recovered in yeast extract agar.

In Fig. 1 survivor curves for *C. botulinum* 62A spores at 104.4°C are shown; these illustrate how spore recovery in pork infusion BT agar (with sodium bicarbonate and sodium thioglycolate) resulted in a large increase in the number of survivors recovered at each heating time compared with spores recovered in pork infusion agar. These survivor curves are typical of the survivor curves for the other experiments listed in Table 2 in that the intercept ratios are less than 1.

At the three test temperatures the D-values for the 62A spores recovered in media without bicarbonate and thioglycolate were all smaller than those for the 62A spores recovered in media containing these reagents. The differences between the D-values were more apparent at 104.4 and 115.6°C than at 121.1°C.

The D-values obtained for 62A spores using modified yeast extract agar (no bicarbonate and thioglycolate added) as the recovery medium were similar to those for 62A spores using pork infusion agar as the recovery medium.

The colonies in yeast extract agar were more easily counted than the colonies in pork infusion agar. The colonies that formed in yeast extract agar were visibly distinct, whereas in some plates the colonies in pork infusion agar were smaller and difficult to see clearly.

The frequency of spreaders in pork infusion agar was greater than in yeast extract agar. The spreaders in yeast extract agar were the result of gas formation in the medium, which rarely occurred. The spreaders in the pork infusion agar appeared to be aided by the presence of small pork particles or precipitated matter in the medium that imparted an opaqueness to the medium and made colony counting difficult.

TABLE 1. *D- and z-values and intercept ratios for C. botulinum 62A, A16037, and B15580 spores heated in Sorensen's 0.067 M phosphate buffer (pH 7) and recovered in yeast extract agar or pork infusion agar*

Spore strain	Recovery medium and z-value (°C)	Test temp (°C)	D-value (min)	95% CI[a] (min)	IR[b]
62A	Yeast extract agar $z = 10.5$	121.1	0.29[c]	0.24–0.36	0.78
		115.6	0.81[d]	0.74–0.90	0.88
		115.6	0.75	0.69–0.82	0.91
		115.6	0.80	0.73–0.89	0.90
		110.0	2.89[e]	2.55–3.34	0.87
		110.0	2.72	2.34–3.21	0.89
		104.4	9.38[f]	8.02–11.29	0.93
		104.4	10.71[g]	9.37–12.51	0.93
		104.4	10.48	9.19–12.21	0.93
62A	Pork infusion agar $z = 11.9$	121.1	0.27[c]	0.20–0.42	0.69
		115.6	0.60[d]	0.56–0.66	0.83
		110.0	1.71[e]	1.37–1.95	0.81
		104.4	6.76[f]	5.68–8.34	0.91
		104.4	6.17[g]	4.61–9.30	0.84
A16037	Yeast extract agar $z = 11.0$	121.1	0.44	0.40–0.49	0.79
		121.1	0.60	0.54–0.67	0.83
		121.1	0.51	0.46–0.56	0.84
		115.6	1.35[h]	1.18–1.57	0.96
		115.6	1.27[i]	1.14–1.44	0.96
		110.0	4.29[j]	3.83–4.88	0.94
		110.0	4.47	3.88–5.23	0.98
		104.4	16.19[k]	15.26–17.25	1.00
		104.4	19.10	17.69–20.77	0.98
A16037	Pork infusion agar $z = 12.8$	115.6	0.85	0.74–1.00	0.81
		115.6	0.98[h]	0.89–1.09	0.87
		115.6	0.93[i]	0.80–1.10	0.77
		110.0	1.80	1.63–2.00	0.87
		110.0	1.84	1.63–2.10	0.83
		110.0	1.74	1.53–2.03	0.81
		110.0	2.18[j]	1.86–2.62	0.82
		104.4	6.98	6.30–7.83	0.89
		104.4	7.86	6.42–10.14	0.90
		104.4	5.99[k]	5.62–6.41	0.93
B15580	Yeast extract agar	110.0	1.37	1.28–1.47	0.98
		110.0	1.34	1.23–1.48	1.01
B15580	Pork infusion agar	110.0	0.53	0.41–0.65	0.88

[a] CI, Confidence interval for the *D*-value.
[b] IR, Intercept ratio ($\log Y_0/\log N_0$).
[c–k] *D*-values with identical superscript letters were from tests run in parallel.

The presence of sodium bicarbonate and sodium thioglycolate in the pork infusion BT agar did not appear to affect the size or clarity of colonies. The only observed difference between pork infusion BT agar and pork infusion agar was the change in colony count for the same dilution.

DISCUSSION

Andersen (2), Treadwell et al. (13), and Wynne et al. (16) have confirmed an essential or stimulatory role of sodium bicarbonate or carbon dioxide for germination of *C. botulinum* spores. In all of these studies, media with bicarbonate resulted in significantly higher counts than media without bicarbonate.

Pork infusion agar is considered to be one of the better media for subculturing heated spores of putrefactive anaerobes (e.g., *C. botulinum*) to obtain spore counts (11), but sodium bicarbonate and sodium thioglycolate are not always included as ingredients.

The National Canners Association's laboratory manual indicates that beef heart, Yesair's pork medium, liver broth, and tryptone-glucose-yeast extract broth are satisfactory for

TABLE 2. *Parallel survivor curve tests with C. botulinum 62A spores heated in Sorensen's 0.067 M phosphate buffer (pH 7)*

Test no.	Test temp (°C)	Yeast extract agar[a]			Pork infusion BT agar[a]			Pork infusion agar[b]			Modified yeast extract agar[b]		
		D-value (min)	95% CI[c] (min)	IR[d]	*D*-value (min)	95% CI (min)	IR	*D*-value (min)	95% CI (min)	IR	*D*-value (min)	95% CI (min)	IR
1	121.1	0.29	0.24–0.36	0.78	0.33	0.26–0.44	0.71	0.27	0.20–0.42	0.69	0.26	0.21–0.33	0.62
2	115.6	0.81	0.74–0.90	0.88	0.78	0.69–0.91	0.90	0.60	0.56–0.66	0.83	0.50	0.46–0.56	0.89
3	104.4	9.88	8.02–11.29	0.93	10.13	8.25–13.10	0.90	6.76	5.68–8.34	0.91	6.19	5.07–7.93	0.93
4	104.4	10.71	9.37–12.51	0.93	10.33	9.08–11.99	0.93	6.17	4.61–9.30	0.84			

[a] Medium contains sodium bicarbonate and sodium thioglycolate.
[b] Medium does not contain sodium bicarbonate or sodium thioglycolate.
[c] CI, Confidence interval for the *D*-values.
[d] IR, Intercept ratio ($\log Y_0/\log N_0$).

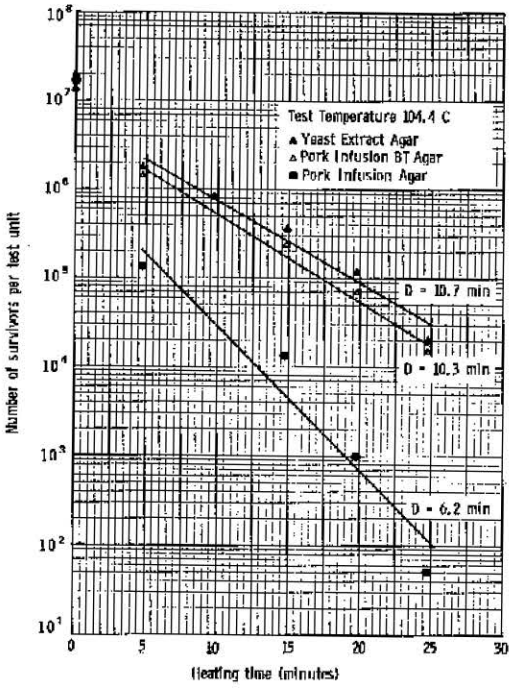

FIG. 1. *Survivor curves for C. botulinum 62A spores heated in Sorensen's 0.067 M phosphate buffer (pH 7). Data are from test 4 in Table 2.*

subculturing heated *C. botulinum* spores (7). None of these media formulations requires sodium bicarbonate or sodium thioglycolate. However, they do mention that 0.1 to 0.2% sodium bicarbonate can be added to make the medium more favorable for rapid germination of *C. botulinum* spores and that 0.1% sodium thioglycolate will improve anaerobic conditions.

The results in this study indicate that to obtain maximum colony counts, the medium used to recover *C. botulinum* spores in thermal resistance survivor curve studies should contain sodium bicarbonate and sodium thioglycolate. Since yeast extract agar is easier to prepare and less expensive than modified pork infusion agar, we use yeast extract agar routinely in our laboratory for thermal resistance studies with *C. botulinum* spores.

ACKNOWLEDGMENTS

The technical assistance of Angela Jones and Yvonne Heisserer is gratefully acknowledged.

This work was supported in part by contract 223-73-2200, Department of Health, Education, and Welfare, Food and Drug Administration.

LITERATURE CITED

1. **Alderton, G., K. A. Ito, and J. K. Chen.** 1976. Chemical manipulation of the heat resistance of *Clostridium botulinum* spores. Appl. Environ. Microbiol. 31:492–498.
2. **Andersen, A. A.** 1951. A rapid plate method of counting spores of *Clostridium botulinum*. J. Bacteriol. 62:425–432.
3. **Augustin, J. A. L., and I. J. Pflug.** 1967. Recovery patterns of spores of putrefactive anaerobe 3679 in various subculture media after heat treatment. Appl. Microbiol. 15:226–276.
4. **Esty, J. R., and K. F. Meyer.** 1932. The heat resistance of the spores of *B. botulinus* and allied anaerobes. XI. J. Infect. Dis. 31:650–663.
5. **Grecz, N., and T. Tang.** 1970. Relation of dipicolinic acid to heat resistance of bacterial spores. J. Gen. Microbiol. 63:303–310.
6. **Kaplan, A. M., H. Reynolds, and H. Lichtenstein.** 1954. Significance of variations in observed slopes of thermal death time curves for putrefactive anaerobes. Food Res. 19:173–181.
7. **National Canners Association.** 1968. Laboratory manual for food canners and processors, vol. 1, Microbiology and processing. AVI Publishing, Westport, Conn.
8. **Odlaug, T. E., and I. J. Pflug.** 1977. Thermal destruction of *Clostridium botulinum* spores suspended in tomato juice in aluminum thermal death time tubes. Appl. Environ. Microbiol. 34:23–29.
9. **Perkins, W. E., D. H. Ashton, and G. M. Evancho.** 1975. Influence of the z-value of *Clostridium botulinum* on the accuracy of process calculations. J. Food Sci. 40:1189–1192.
10. **Pflug, I. J., and R. G. Holcomb.** 1977. Principles of thermal destruction of microorganisms, p. 933–994. *In* S. S. Block (ed.), Disinfection, sterilization and

preservation. Lea and Febiger, Philadelphia.
11. **Stumbo, C. R.** 1973. Thermobacteriology in food processing, 2nd ed., p. 29–30. Academic Press Inc., New York.
12. **Stumbo, C. R., J. R. Murphy, and J. Cochran.** 1950. Nature of thermal death time curves for PA3679 and *Clostridium botulinum*. Food Technol. 9:321–326.
13. **Treadwell, P. E., G. J. Jann, and A. J. Salle.** 1958. Studies on factors affecting rapid germinating of spores of *Clostridium botulinum*. J. Bacteriol. 76:549–556.
14. **Tauji, K., and W. E. Perkins.** 1962. Sporulation of *Clostridium botulinum*. I. Selection of an particulate sporulation medium. J. Bacteriol. 84:81–85.
15. **Wheaton, E., and G. B. Pratt.** 1961. Comparative studies on media for counting anaerobic bacterial spores. II. J. Food Sci. 26:261–268.
16. **Wynne, E. S., W. R. Schmieding, and G. T. Daye, Jr.** 1955. A simplified medium for counting *Clostridium* spores. Food Res. 20:9–12.
17. **Xezones, H., and I. J. Hutchings.** 1965. Thermal resistance of *Clostridium botulinum* (62A) spores as affected by fundamental food constituents. Food Technol. 19:113–115.