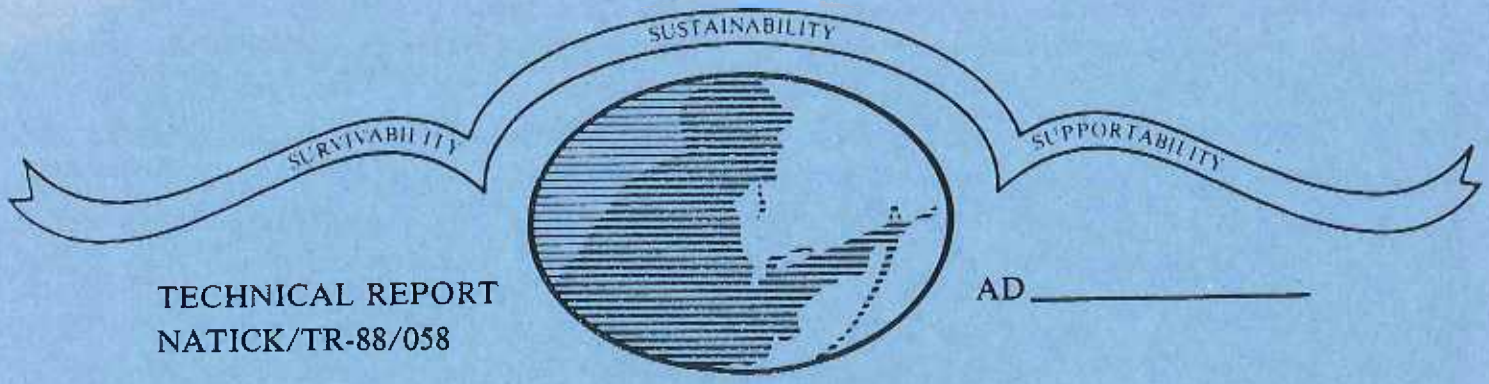

TECHNICAL REPORT
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PRODUCTION OF SPORES OF PA3679 OF HIGH HEAT RESISTANCE AND HIGH YIELD IN A BIPHASIC BEEF HEART INFUSION MEDIUM

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A method is described for the production of spores of Clostridium sporogenes strain PA3679 using a biphasic beef heart medium. These spores were produced in high yield and exhibited a higher heat resistance than any reported to date in the literature.

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PREFACE

A reproducible method for the production of sufficient numbers of heat resistant spores and clean spores of Clostridium sporogenes strain 3679 (PA3679) for conducting inoculated pack studies is of great value. The biphasic sporulation technique has been successfully employed for the production of Clostridium botulinum spores. This technique produced sufficient numbers of clean C. botulinum spores. The procedures employed by other investigators to produce PA3679 spores yielded either low numbers of spores, spores which had lower D-values, or spores which could not be separated from the meat particles in the medium. This study reports the successful adaptation of the biphasic technique for the production of clean PA3679 spores of extremely high thermal resistivity.

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PRODUCTION OF SPORES OF PA3679 OF HIGH HEAT RESISTANCE AND HIGH YIELD IN A BIPHASIC BEEF HEART INFUSION MEDIUM.

INTRODUCTION

Spores of Clostridium sporogenes strain PA3679, are among the most resistant of food spoilage organisms, and are used as nontoxic surrogate test organisms in lieu of Clostridium botulinum, to evaluate thermal processing in low-acid canned foods. It is often necessary to challenge a particular product with an inoculated pack study in order to confirm preliminary results obtained with resistance studies conducted in buffer or in food materials with subsequent recovery in optimal growth medium, or on a small scale using small quantities of food materials. The capability of producing clean spores of PA3679 having the required resistance in order to conduct an inoculated pack study is therefore of great importance.

A number of studies have been conducted to produce spores of adequate resistivity. Several investigators have reported the use of pork infusions[1] and beef heart infusions[2] to produce high yields of thermally resistant spores. Goldoni et al.,[3] using beef heart infusion medium, reported the production of spores with acceptable D-values and Z-values. Production of spores, in a meat-based liquid medium[3] results in a mixture of spores, vegetative cells and particulate debris from which it is extremely difficult to obtain a clean suspension of spores without an appreciable loss in spore yield.

Fermentors have been used to obtain clean spores.[4, 5] In these studies spores of PA3679 were grown in more defined conventional media without food particles, with different concentrations of glucose, and with or without pH control. Although thermal resistivity varied widely, clean spores were obtained.

The use of biphasic methods to produce spores of Clostridium botulinum has been employed in the authors' laboratory and by others.[2, 4] In this study, the beef heart medium of Goldoni et al.[3] was adapted for use in a biphasic medium and coupled to an anaerobic gassing technique[2] for the production of spores of PA3679 which are clean and extremely heat resistant.

METHODS

Preparation of beef heart infusion broth (BHIB): The preparation of BHIB was based on the method described by Goldoni et al.[3] The mixture was heated to boiling and simmered for 1.5 to 2 hours, cooled to room temperature, and refrigerated overnight. Congealed fat was removed and the liquid was decanted from the meat particles, which were retained for later addition to the complete medium. The broth was clarified by filtration through filter paper in a Buchner funnel. To each liter of infusion, 10 g of tryptone, 10 g of gelatin (Difco), 0.5 g of glucose, 4 g of dibasic potassium phosphate, 3 g of sodium citrate, and 15 g of skim milk powder were added.[3] The pH was adjusted to 8.5. This medium (BHIB) was dispensed into tubes in volumes of 7 mL and 18 mL, and into 500-mL flasks in volumes of 200 mL, and then sterilized. In Fernbach flasks (2800 mL) a 2-cm layer of meat particles, derived from the infusion preparation, was placed over the bottom of the flask. The particles were then overlaid with beef heart infusion agar (BHIA) prepared from 750 mL of BHIB containing 1.5% agar. The Fernbach flask and its contents was sterilized, and if not used immediately, was stored in an anaerobic hood (Coy Laboratory Products, Inc., Ann Arbor, MI).

Inocula were prepared by an active culture technique reported by Goldoni et al.[3]. A 7-mL tube of BHIB was inoculated with heat-activated spores of PA3679 (Tech S Corp., Washington, D.C.; 30 minutes at 80°C) and incubated at 30°C for 18 hours. Two tubes, each containing 18 mL of

BHIB, were inoculated with 2 mL from the 18-hour culture and incubated at 30°C for 4 hours. The entire contents were used to inoculate 150 mL of BHIB which was incubated at 30°C for 3 hours. Two of the 3-hour cultures were used to inoculate the Fernbach flask containing BHIA. The 300 mL of inoculum overlaid the agar base and constituted the biphasic culture. Each Fernbach was equipped with a sterile rubber stopper equipped with inlet and outlet tubing to allow flushing of head space with sterile nitrogen for 30 minutes before incubation. Flasks were incubated at 30°C for 21 days. The liquid phase (ca. 300 mL including rinse water) was centrifuged, the pellet was washed 3 times with distilled water, resuspended in distilled water, transferred quantitatively to a 100-mL volumetric flask, and adjusted to the desired spore concentration.

Determination of D-values: Heat resistance studies were performed in the BIER (Biological Indicator Evaluator Resistometer, Joslyn Valve, Inc., Macedon, NY), using aqueous suspensions of PA3679 spores which had been activated at 80°C for 30 minutes. The spore suspension was placed in cups made from trimmed down stainless steel Morton test tube closures, 16 mm in diameter x 12 mm high. A series of five replicate cups containing 0.5 mL of spore suspension were exposed for various periods of time to temperatures of 240°F, 245°F and 250°F. At the completion of the heating cycle, the cups were removed and immediately dropped into 9.5 mL of chilled sterile 0.02 M phosphate buffer, pH 7.0. Serial dilutions were made, 1.0 mL of each appropriate dilution was added to a sterile test tube, and 8 mL of pork infusion agar (PIA)[7] recovery medium was poured

into the tube containing the heat-treated spore sample. The preparation of PIA, modified to include steps to eliminate cloudiness due to finely suspended particulates that obscure colony enumeration, will be reported elsewhere. An overlay of 2% agar containing 0.5% sodium thioglycolate was added to the top of each tube to ensure anaerobiosis. The tubes were incubated at 30°C for 5 days and the colonies were counted. The survival curve was plotted on semilogarithmic graph paper, and the slope of the least squares regression line of the linear portion of the survival curve was determined.[8] The D-values were calculated by taking the negative reciprocal of the slope of the regression line.

RESULTS AND DISCUSSION

Table 1 compares data obtained in this laboratory with published reports on the yield and heat resistance of spores of PA3679 produced in various types of growth media by stationary cultures or in fermentors. If D-value at the specific temperatures (T_2) of 240°F, 245°F and 250°F was not reported in a study, then the reported D-values (D_{T_2}) were converted to their equivalent values (D_{T_1}) at the desired temperature (T_1) according to equation 1. Where no specific Z-value was reported, a value of 18°F was used.

$$D_{T_1} = D_{T_2} \times 10^{\frac{T_2 - T_1}{Z}} \qquad (1)$$

The D-values given in Table 1 are the highest obtained in each study. A comparison of thermal resistivity (Table 1) demonstrates that the presence of meat infusion is not necessary for the formation of acceptably resistant spores (D_{240} = 2.4 min.[3]) Spores produced in our laboratory in beef heart infusion medium, using the method of Goldoni et al.[3], had essentially the same yield and the same D- and Z-values as theirs. Note that the method of Goldoni et al.[3] uses 0.5 g/L of glucose, while the concentration of glucose used by others was considerably higher.[4, 5] The yield of spores in a pork infusion biphasic medium was reported to be proportional to the glucose concentration, whereas heat resistance was inversely proportional.[1]

TABLE 1. Comparison of Heat Resistance of *Clostridium sporogenes* strain 3679 Spores Prepared by Different Methods.

Method	pH	D-value[a] (minutes) 230°F	240°F	245°F	250°F	Z[b] (°F)	Yield (x10^6/mL)	Glucose (g/L)	Reference
Beef heart infusion Biphasic, flask	u[c]	(31.4)[d]	9.39	5.03	2.80	19.1	14.0	0.5	[e]
Beef heart infusion Broth, flask	u	-	4.56	(2.58)	(1.46)	20.2	1.6	0.5	Goldoni, 1980
Beef heart infusion Broth, flask	u	-	5.52	3.20	1.78	20.5	3.4	0.5	[e]
Pork infusion Biphasic, flask	u	14.08	5.58[f]	1.75[g]	-	18.94	17.4[h]	5.0	Ababouch, 1985
Pork infusion Biphasic, flask	c	9.75	5.08[f]	2.77[g]	-	17.39	14.0	5.0	Ababouch, 1985
Trypticase-Soy broth Fermentor	c	20	(5.80)	(1.70)	-	18.25	40.0	15.0	Pang, 1983
Tryptone-Salt broth Fermentor[i]	u	80	-	-	-	-	75.0	5.0	Anema, 1973
Tryptone-Salt broth Fermentor[i]	c	13	-	-	-	-	75.0	5.0	Anema, 1973
Unknown[j]	-	-	(5.39)	(2.84)	1.50	18.0	-	-	[e]

[a] D-values = minutes per log decrease in survivors. Where more than one D-value was applied the highest D-values were used.
[b] Z-value = degrees (°F) per log decrease in D-value.
[c] u = uncontrolled pH; c = controlled pH.
[d] Values in parentheses are calculated from D- and Z-values reported in reference.
[e] Results by the authors', 1988.
[f] Temperature of 239°F was used.
[g] Temperature of 247°F was used.
[h] Average of two experiments of 4.9×10^6 and 3.0×10^7 spores/mL.
[i] *Clostridium sporogenes* strain BC_2 was used, not PA3679.
[j] Spores obtained from Tech S Corp., Washington D. C. Methods of preparation and yield not known.

The use of a fermenter, regardless of whether the pH is controlled or not, does not result in more highly resistant spores but does increase yield. The D_{110} values reported by Pang et al.[5] varied from spores having too low a resistance for use in thermal resistance studies to spores with sufficient resistance. Although no Z-values were given, Anema et al.,[4] using a tryptone-salt medium, obtained highly resistant spores with a D_{110} of 80 minutes from medium containing 0.5% glucose, but spores of none or low resistance from medium containing 0%, 0.2%, or 1% glucose. At controlled pH, for all of these glucose concentrations, the D_{110} was consistently 13 minutes. The extremely high D_{110} value reported for spores obtained by Pang et al.[5] with 0.5% glucose has not been equalled by the other investigators and appears to be atypical of other studies.

The spores obtained in this study (Table 1) with the biphasic technique using beef heart infusion broth, yielded the most resistant spores of PA3679, with the exception of that obtained under the one condition noted above by Anema et al.[4] Table 2 shows the consistency in the D-values displayed by different spore preparations produced over a period of 18 months. In the

TABLE 2. Thermal resistance of three biphasic preparations of Clostridium sporogenes strain 3679.

Preparation	D-value (minutes) 240 °F	245 °F	250 °F
1		5.00	
2		5.01	
	9.40	5.03	2.82
3	9.39	5.04	2.78
	10.81	4.73	2.80

temperature range of 240 to 250°F, these spores are 50% to 100% more resistant than those obtained by other techniques. Ababouch and Busta[4] experienced large variations in thermal resistivity between biphasic spore crops, although the variability was least for spores grown with a glucose concentration of 2 g/L and 5 g/L. As seen in Table 2, the D-values of the three spore preparations used in this study were in very close agreement, having less than a 10% difference in D-values. As seen in Figure 1 the thermal inactivation kinetics are exponential, and it is quite apparent that at 240°F spore activation[9] prior to inactivation occurs. That these activated spores,

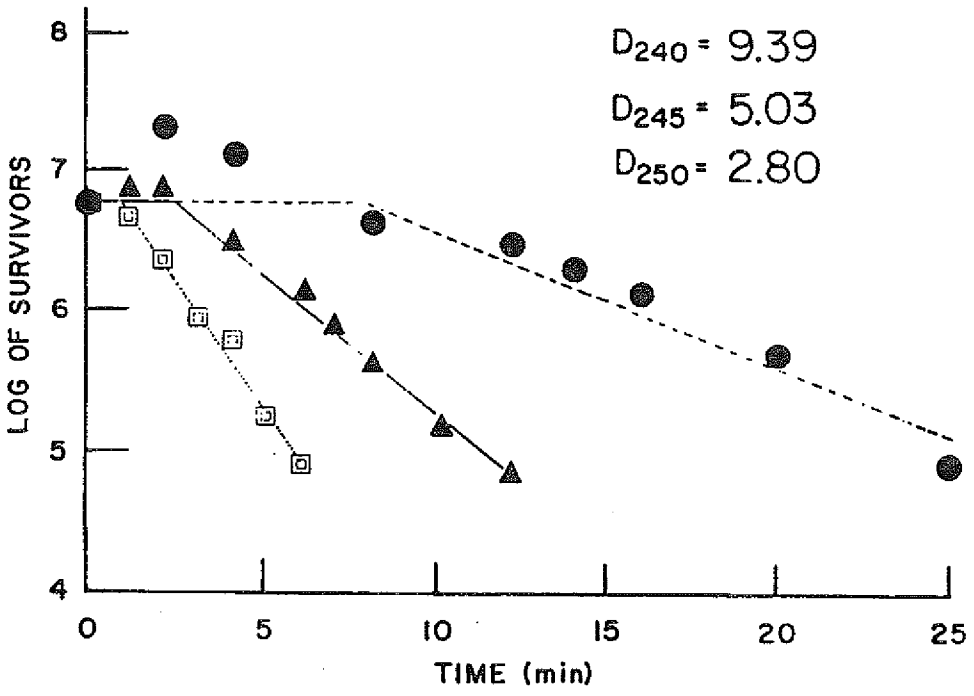

Figure 1. Survival curves of PA3679 spores at 240°F, 245°F, and 250°F. Legend: ●-●, 240°F; ▲-▲, 245°F; ▣-▣, 250°F.

shown by an increased viable spore concentration at two and four minutes, are not unique is indicated by the fact that their inactivation slope is an extension of the main curve. As shown in Figure 2, the Z-value obtained is 19.05°F.

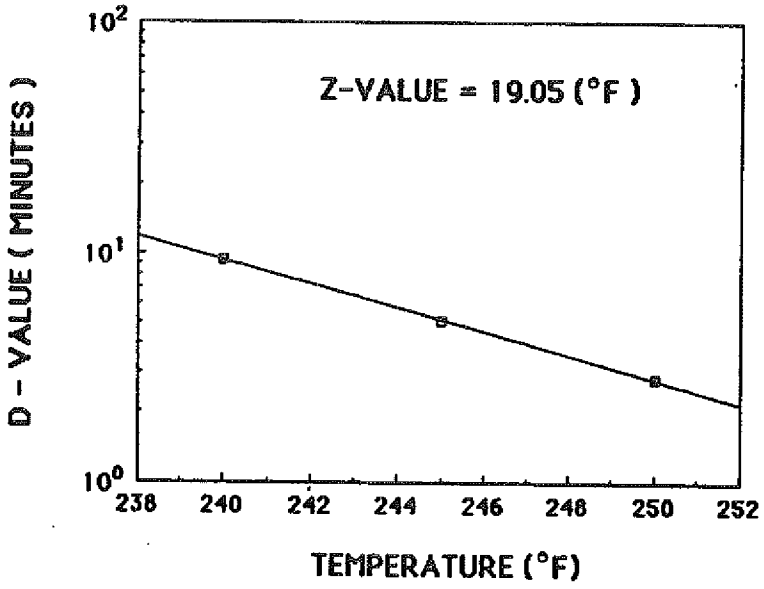

Figure 2. Thermal death time curve of PA3679 spores.

As noted by Pang et al.,[5] spore crops having different thermal resistances can be useful for designing processes of specific lethalities or for products more susceptible to heat. In some instances, when studying aseptic processing, one may require spores having extremely high D-values. Of equal importance in validating thermal processes is obtaining spore crops with desirable Z-values. As seen in Table 1, and it is also evident in the more detailed data presented in the appropriate references, a variety of D- and Z-values can be obtained for application to different thermal processing needs. Verification of whether these differences in thermal resistivity can be consistently produced is necessary.

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