Copyright © 2003 by Humana Press Inc.
All rights of any nature whatsoever reserved.
0273-2289/03/105-108/0287/$20.00

Effect of Media on Spore Yield and Thermal Resistance of *Bacillus stearothermophilus*

Thereza Christina Vessoni Penna,*
Irene A. Machoshvili, and Marina Ishii

Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Science, University of São Paulo, Rua Antonio de Macedo Soares, 452 04607-000, São Paulo/SP, Brazil, E-mail: tcvpenna@usp.br

Abstract

The interference of eight components in the yield of sporulation and thermal resistance to moist heat (121°C) of *Bacillus stearothermophilus* spores suspended in 0.02 *M* calcium acetate solution and inoculated on paper strips previously treated with calcium acetate/calcium hydroxide was studied. The spore yield of 1.0×10^8/mL was developed at 62°C in 17 media containing different concentrations of D-glucose, sodium chloride, L-glutamic acid, yeast extract, peptone, manganese sulfate, potassium phosphate, and ammonium phosphate. The combined effects of yeast extract, peptone, and glucose contributed positively to the spore yield and to the stability of the thermal resistance of both spores in suspension and on strips.

Index Entries: *Bacillus stearothermophilus*; thermoresistance; sporulation; bioindicator.

Introduction

The length of the exponential growth phase determines the final cell population of *Bacillus stearothermophilus*. Symmetrical cell division depends on favorable exogenous conditions such as temperature, humidity, nutrients, and aeration. The sporulation of *B. stearothermophilus* is characterized by a multiphase, morphologic synchronized process that occurs at the onset of the stationary phase *(1)*. During the maturation of the spore, thermoresistance characteristics develop. Although the complex morphologic changes that occur during sporulation result from regulated changes in gene expression, the thermal resistance of mature spores can be manipulated in vitro *(2–4)*. Spores in suspension, when treated with sodium, potassium, magnesium, and manganese cations, are more resistant than spores

*Author to whom all correspondence and reprint requests should be addressed.

Table 1
Media Used for Sporulation of *B. stearothermophilus* ATCC 7953 to Evaluate Interference of Eight Components in Thermal Resistance of Developed Spores

	Media concentrations (% w/v)																
Components	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
D(+)Glucose	0.25	0.25	0.018	0.25	0.018	0.018	0.018	0.25	0.018	0.018	0.25	0.018	0.25	0.018	0.25	0.018	0.25
L-Glutamic acid	0.04	0.04	0.04	0.04	0.04	0.04	—	—	—	—	—	—	—	—	—	—	—
Yeast extract	0.4	0.4	0.4	0.05	0.05	0.4	0.4	0.4	0.4	0.05	0.05	0.05	0.05	0.4	0.4	0.4	0.4
Peptone	0.5	0.3	0.3	0.3	0.5	0.5	0.3	0.5	0.5	0.3	0.3	0.5	0.5	0.3	0.3	0.5	0.5
NaCl	0.001	0.001	0.001	0.001	0.001	0.001	1.0	1.0	1.0	—	—	—	—	—	—	—	—
$MnSO_4$	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	—	—	—	—	—	—	—	—
$NH_4H_2PO_4$	—	—	—	—	—	—	0.035	0.035	0.035	—	—	—	—	—	—	—	—
KH_2PO_4	—	—	—	—	—	—	0.05	0.05	0.05	—	—	—	—	—	—	—	—
Agar	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
pH	9.84	10.02	9.30	9.50	9.50	9.05	9.63	9.10	9.4	9.4	9.6	9.6	9.6	9.6	9.6	9.6	9.6

[a]The final pH of the medium was adjusted to 9.7 ± 0.1 with $Ca(OH)_2$. L-Glutamic acid was added to the medium following autoclaving at 121°C for 30 min of the other components dissolved in water.

in the hydrogenionic form. The hydrogen form of the spores can be converted in a calcium-resistant form by treatment with calcium acetate (CaAc) at alkaline pH *(2)*. Acid conditions of the heating menstruum may cause partial demineralization of the spores *(3)*. On the other hand, the mineralization provides a reduction in the content of water in the protoplast of the spore, provoking an increase in thermal resistance *(4)*. When inoculated on strips previously treated with 0.02 *M* CaAc, the *B. stearothermophilus* ATCC 7953 spores displayed greater stability *(5)*.

Mineralization of the cortex depends either on the supply of the sporulation medium or on the suspension of harvested spores *(6)*. Thermal resistance is associated with the peptidoglycan present in the spore cortex. At low pH, the acid protonates the peptidoglycan, decreasing thermal resistance and the viability of spores. Therefore, the pH of the culture medium influences spore yields, which at pH 8.7 were higher than those obtained in media at pH values between 7.0 and 5.5 *(6)*.

Spores of *B. stearothermophilus* are mainly used as a bioindicator (BI) to monitor and ensure the reproducibility of moist heat processes *(7,8)*. Bioindicator's are commercially available in suspension form, for impregnation in the load unit; inoculated on paper carriers; or in a self-contained ampoule where spores are suspended in a recovery medium. Strips of paper, which are inexpensive and small, are easily placed in strategic positions in order to verify the homogeneity of the sterilant distribution in the chamber and in the unit of the load.

The purpose of the present study was to evaluate the interference of eight components in the yield of sporulation and thermal resistance to moist heat (121°C) of *B. stearothermophilus* spores in a suspension of 0.02 *M* CaAc and on strips previously treated with CaAc/calcium hydroxide ($Ca[OH]_2$) (pH 11.0) *(9)*. The principal goal was to verify which component, individually or in association, showed the greatest influence on viability and thermal resistance.

Materials and Methods

Culture Media

Sterile techniques were carried out in a class 100 flow cabinet and the same lots of component (Sigma, St. Louis, MO) and culture media (Difco) were used: Plate count agar (PCA), (0.5% tryptone, 0.25% yeast extract, 0.1% dextrose, and 1.5% agar at pH 7.0 ± 0.2), Trypticase soy broth (TSB), (1.7% tryptone, 0.3% soytone, 0.25% dextrose, 0.5% NaCl, and 0.25% dipotassium phosphate at pH 7.3 ± 0.2) and Trypticase soy agar (TSA), 1.5% tryptone, 0.5% soytone, 0.5% NaCl, and 1.5% agar at pH 7.3 ± 0.2).

Spore Production

B. stearothermophilus ATCC 7953 spore suspension cultures were developed in 17 media (Table 1) containing the respective components and concentrations (% [w/v]): D-glucose (0.018–0.25%), NaCl (0.001–1.0%), L-glu-

tamic acid (0.04%), yeast extract (0.05–0.4%), peptone (0.3–0.5%), manganese sulfate (0.001%), potassium phosphate (0.05%), and ammonium phosphate (0.05%). After 24 h at 62°C, smooth morphotype variant single colonies (small and transparent) were harvested from PCA tube slants, resuspended in 0.1% peptone salt fluid, and each 5-mL suspension was heat shocked at 120°C for 5 min. Each 200-mL medium (Roux flask) was inoculated with 10 mL of activated suspension. After 6 d at 62°C saturated humidity, growth was harvested in 0.02 *M* CaAc, and the suspension was adjusted to pH 9.7 with 0.14% $Ca(OH)_2$ and centrifuged (1935*g* for 30 min) four times. The spore pellets were suspended in chilled 0.02 *M* CaAc (pH 9.7), and stored at 4°C *(8)*. The viability of 10^7–10^8 colony-forming units(CFU)/mL (average CFU spores/mL) of heat-shocked spores in suspension (97.8°C for 30 min) was estimated through pour plates in TSA at 62°C for 48 h, from a minimum of ten plates.

Preparation of Bioindicators Formed by Spores Impregnated on Strips (6)

Sterile paper strips (250 g/m^2, 7 mm wide, 0.2 mm deep, 20 mm long) were immersed in 0.02 *M* CaAc (pH 11/ 0.14% $Ca[OH]_2$) for 12 h to a final pH of 9.0 ± 0.1, dried at 45°C for 24 h and stored at –18°C. The monolayer disposed strips were individually inoculated with 0.1 mL of *B. stearothermophilus* homogenized suspension (pH 9.7). The impregnated strips (bioindicators) were dried at 45°C for 24 h and stored at –18°C. Strip spore quantification was performed by blending 10 strips in 100 mL of 0.1% peptone salt fluid. Standard pour plate enumeration in TSA (62°C for 48 h) was used for initial heat shocked (97.8°C for 30 min) spore-per-strip density determination.

Thermal Treatments

Thermal treatments at 121°C were performed through the serum bottle technique apparatus *(5,9)*. The homogenized spore suspensions (5 mL) were transferred into 20-mL glass serum bottles (Wheaton SB205A; 60 × 25 mm). Ten *B. stearothermophilus* spore strips, previously humidified by instantaneous contact with sterile distilled water, were transferred to 20-mL serum bottles and placed between 10 Durham tubes (30 × 3 mm). The distance between neighboring parallel tubes was 4.0 ± 0.5 mm, and each Durham tube was filled with water to two thirds its total capacity. The bottles were closed with a sterile flange rubber stopper and then sealed with an aluminum seal.

Air was removed (5 min) by suction (General Electric vacuum pump, 1725 rpm, 1/6 Hp), with a 22-gage stainless steel needle (30 × 7 mm) through the rubber stopper of the sealed bottles. A thermocouple sensor end, inside a 316 stainless steel needle (1.5 × 100 mm), was placed in the geometric center of the bottles, using the hole through the rubber stopper left by the pump needle. Triplicate sets of bottles were heated in a thermostatically

controlled oil (Dow Corning® silicone 200/220 CS, P = 0.948 g/cm^3) bath (OTB-7A, Haven Automation) at different time intervals at 121.0 ± 0.1°C and were cooled and held in an ice-water bath. The experimentally determined lag correction of 7 min was used. The thermocouples, type J (2x32AWG), overwrapped with Teflon, were equilibrated at 121.0 ± 0.1°C and attached to multipoint recording equipment (IOPE therm 400-CE 12; recording rate: 30 s). Survivors on the strips were quantified for spore viability and expressed by decimal logarithms of the average colony-forming units per strip, from at least 10 plates for each time heating condition and system used.

Determination of Decimal Reduction Times

The decimal reduction times (D value, the time in minutes to kill 90% of the initial spore population at 121°C) were determined from the negative reciprocal of slopes (b_1) of the regression lines, using the linear portions of the survivor curves ($\log_{10}$ survivors vs time). The angular coefficient standard error SE (b_1) and the multiple determination coefficient (R^2) were also calculated. The estimated value of the D value standard error SE(D) was obtained from the root of the variance $(V[D])^{1/2}$, which was calculated as follows:

$$V(D) = ([1/b_1^2]^2 \times [\mathrm{SE}\{b_1\}]^2)$$

The upper (D – mean + SE[D]) and lower (D – mean – SE[D]) D values were also expressed.

Results and Discussion

Seventeen different media were used to examine, simultaneously, the influence of cations (Ca^{++}, Mg^{++}, NH_4^+, K^+, Mn^{++}, Na^+) and the concentration of D-glucose, peptone, and yeast extract on the formation of *B. stearothermophilus* spores and their thermal resistance. The initial population of the activated spores varied from 1.0×10^7 to 5.0×10^8 spores/mL of 0.02 *M* CaAc (Table 2). The pH of the CaAc suspension varied from 7.32 to 7.80 after 1 mo storage at 6 ± 1°C. Following readjustment with $Ca(OH)_2$, the pH was equilibrated between 9.30 and 10.02, where it remained stable.

For an overkill in moist heat sterilization process (total time = 12 d), BIs containing ≥5 × 105 spores of *B. stearothermophilus*/carrier should present a $D_{121°C}$ value of about 1.9 min *(7)*. Therefore, the thermal resistance of the bioindicators's formed by spores in suspension and by spores on strips can be classified (95% confidence), according to $D_{121°C}$ values, into three groups: group 1 ($D_{121°C} < 2.0$ min); group 2 (2.0 min ≤ $D_{121°C}$ ≤ 2.46 min); and group 3 ($D_{121°C} > 2.46$ min).

In group 1, spores developed in media 4 and 8 showed a very close average $D_{121°C}$ for both BIs in suspension (1.76 and 1.71 min) and on strips (1.82 and 1.87 min). In medium 4, the maximum glucose concentration was incorporated with the minimum concentrations of yeast extract, peptone,

Table 2
Decimal Reduction Times (D values, min)
at 121°C for Spores Developed in 1–17 Media[a]

	Adjusted pH		Initial	Spores in suspension $D_{121°C}$ values (min)			Spores on strips $D_{121°C}$ values (min)		
Medium	Before	After	(spores/mL)	Mean	Lower	Upper	Mean	Lower	Upper
1	7.81	9.84	5.0×10^8	2.41	2.32	2.51	2.0	1.89	2.11
2	6.68	10.0	3.0×10^8	1.54	1.49	1.60	1.94	1.86	2.02
3		9.30	2.0×10^7	2.66	2.54	2.78	1.90	1.82	2.00
4		9.50	3.0×10^8	1.76	1.71	1.82	1.82	1.74	1.91
5		9.50	2.0×10^8	2.71	2.64	2.77	2.34	2.22	2.47
6		9.05	2.0×10^8	2.09	2.04	2.15	1.57	1.51	1.63
7		9.63	2.0×10^8	2.41	2.32	2.50	2.47	2.40	2.53
8		9.10	5.0×10^7	1.71	1.65	1.77	1.87	1.82	1.93
9		9.40	1.0×10^7	2.00	1.94	2.07	1.83	1.74	1.92
10	8.06	9.60	8.9×10^7	1.86	1.80	1.92	2.26	2.18	2.33
11	7.32	9.60	5.1×10^7	1.84	1.76	1.92	2.48	2.37	2.59
12	7.80	9.60	6.0×10^7	2.84	2.73	2.94	2.32	2.19	2.44
13	7.46	9.60	5.0×10^7	2.36	2.27	2.44	2.26	2.18	2.34
14	7.27	9.60	3.4×10^7	2.15	2.07	2.23	2.79	2.59	2.99
15	7.54	9.60	9.5×10^6	2.23	2.10	2.36	2.69	2.54	2.84
16	7.46	9.60	1.4×10^7	2.76	2.62	2.88	3.46	3.28	3.63
17	7.60	9.60	1.0×10^8	2.86	2.68	3.04	2.39	2.25	2.53

[a]$D_{121°C}$ values (min), decimal reduction times; D value, the time in minutes to kill 90% of the initial spore population at 121°C.

and NaCl. By contrast, in medium 8, NaCl at a maximum concentration showed a deleterious effect on the BIs thermoresistance, even in the presence of glucose, peptone, and yeast extract also at maximum concentration. In medium 2, glucose and yeast extract were present in maximum concentrations and peptone and NaCl in minimum concentrations, and the *D* value of 1.54 min for BIs in suspension was the lowest obtained, although the *D* value was 1.94 min for BIs on the strips. With the *D* values of 1.86 and 1.84 min for the spores in suspension, respectively from media 10 and 11, the minimum concentration of yeast extract in the absence of NaCl, was sufficient to maintain the thermal resistance of bioindicators stable on the strips (2.26 and 2.48 min, respectively). The spores on the strips from medium 7 presented the lowest *D* value of 1.57 min, with the maximum concentrations of yeast extract and peptone and minimum concentrations of glucose and NaCl. The negative interference of the maximum concentrations of glucose and NaCl in the thermoresistant properties of the spores confirmed the data obtained for the spores developed in 32 media prepared by 11 components *(9)*. The presence of ammonium phosphate, yeast, and peptone, all at maximum concentrations, did not annul but merely partially canceled the negative effect of NaCl and glucose on the $D_{121°C}$ values.

Group 2 comprised the majority of the BIs in suspension and on strips. In this group, it was observed that the deleterious effect of glucose at maximum concentration (medium 1) was partially neutralized in the presence of yeast and/or peptone. In media 7 and 9, even with glucose at a minimum concentration, the presence of NaCl at a maximum concentration annulled the positive effects of yeast and/or peptone. The presence of the other salts, ammonium phosphate, potassium phosphate, and manganese sulfate revealed no influence on the thermoresistance of the BIs. This fact was confirmed for the spores from media 13–15.

In group 3, the BIs that showed $D_{121°C}$ values >2.46 min for the spores in suspension were developed in media 3, 5, 12, 16, and 17, with $D_{121°C}$ values equal to 2.66, 2.71, 2.84, 2.76, and 2.86 min, respectively, and for the spores on strips from the media 15 and 16, respectively, with $D_{121°C}$ values equal to 2.69 and 3.46 min. Glucose was present at the minimum concentration, with the exception of media 15 and 17. Yeast extract was present at the maximum concentration in media 3, 15, 16, and 17; peptone was incorporated at the maximum concentration in media 5, 12, 16, and 17. Both components were simultaneously incorporated at their respective maximum concentrations in media 16 and 17, where the presence of glucose at the maximum concentration in media 17 interfered only slightly in the thermoresistance of the spores on the strip. Medium 16 showed the best conditions for maturing the spores, providing the nutrients required to confer maximum thermal stability to spores developed over a period of 6 d at 62°C, with saturated humidity.

Peptone at a maximum concentration is an excellent source of mineral salts and amino acids at concentrations required for the formation of mature spores and the maintenance of thermal resistance. Glucose is a

source of carbon that supplies the energy required for the logarithmic phase of development before the stationary growth phase is reached and then forms the spores. Yeast is an excellent source of B complex vitamins essential for growth and sporulation.

The components peptone and yeast at maximum concentrations and glucose at minimum concentration provided good viability, about 1×10^8 spores/mL, with a *D* value at 121°C > 1.5 min for both BIs (the spores in suspension and on strips), matching international standards.

Acknowledgments

This study was made possible by financial support provided by Brazilian Committees for Scientific Technology Research (Conselho Nacional de Desenvolvimento Científico e Tecnológico and Fundação de Amparo à Pesquisa do Estado de São Paulo).

References

1. Foster, S. J. (1994), *J. Appl. Bacteriol. Symp. Suppl.* **76,** 25S–39S.
2. Reich, R. R., Whitbourne, J. E., and McDaniel, A. W. (1979), *J. Parenter. Drug Assoc.* **33,** 228–234.
3. Marquis, R. E. , Sin, J., Shin, and S. Y. (1994), *J. Bacteriol.* **76, (Suppl.)** 40S–48S.
4. Beaman, T. C. and Gerhardt, P. (1986), *Appl. Environ. Microbiol.* **52,** 1242–1246.
5. Vessoni Penna, T. C., Machoshvili, I. A., and Taqueda, M. H. S. (1996), *J. Pharm. Sci. Technol.* **50,** 1–11.
6. Vessoni Penna, T. C., Machoshvili, I. A., and Taqueda, M. E. S. (1998), *J. Parenter. Sci. Technol.,* **52,** 198–208.
7. *United States Pharmacopeia,* 23rd and 24th eds. (1995 and 2000), United States Pharmacopeial Convention, Rockville, MD.
8. Graham, G. S. and Boris, C. A. (1993), In *Sterilization Technology: A Practical Guide for Manufacturers and Users of Health Care Products,* Morrissey, R. F. and Phillips, G. B. eds., Van Nostrand, Reinhold, NY, pp. 36–69.
9. Vessoni Penna, T. C., Machoshvili, I. A., Taqueda, M. E. S., and Ishii, M. (2000), *J. Parenter. Sci. Technol.,* **54,** 398–412.