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STUDIES ON SPORULATION OPTIMIZATION AND CHARACTERIZATION OF *BACILLUS SUBTILIS* SPORE QUALITY

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14. ABSTRACT

Manganese-Amended Nutrient Agar is the media currently used by the U.S. Environmental Protection Agency for the production of *Bacillus subtilis* spores. The cells require 12–14 days to sporulate. Our goal was to conduct a study exploring replacement of the current media to minimize the incubation period and generate high quality *B. subtilis* spores. An extensive literature review was performed to investigate widely accepted methods used for sporulation of *B. subtilis*. Based on the results of this review, three media were investigated: Synthetic Sporulation Medium, 2xSG, and Long Version Nutrient Agar. The selected formulations or close variations have extensively been used by other researchers. These formulations represent two extreme ends of the spectrum (i.e., fully defined to undefined or complex). They have been used depending on specific objectives (i.e., molecular versus spore production). The Synthetic Sporulation media (either agar or broth versions) yielded poor results, with no sporulation of *B. subtilis* during a 14 day monitoring period. Sporulation was completed within 7–9 days of incubation in the other two media. Finally, heat and bleach resistance were recommended in place of acid resistance as indicators of spore hardiness.

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Sporulation *Bacillus subtilis* Complex versus defined media

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PREFACE

The work described in this report was started in November 2006 and completed in January 2008.

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1. INTRODUCTION

Sporulation differentiation, unique to two bacterial species, *Clostridium* and *Bacillus*, is a process induced by reduced levels of nutrients in the environment or in culture (Driks, 2002). Spores can survive for long periods without nutrients or water. In contrast, when vegetative cells are cultured in media for varying periods, nitrogen starvation signals a physiological switch in cells. The media composition used to induce sporulation varies extensively in each laboratory, ranging from a completely synthetic composition at one end of the spectrum to a mostly complex one at the other end.

It is clear that no single standardized spore preparation method is available for producing predictable quality spores of *Bacillus subtilis*. Post harvest treatment of spores also varies from a few simple washes with sterile distilled water to heat ethanol and lysozyme treatments, followed by purification over a gradient column (Tamir and Gilvarg, 1966; Prentice et al., 1972). Furthermore, no common single set of spore quality indicators (i.e., spore yield, heat resistance, acid resistance, or radiation resistance) has consistently been used by different researchers. Heat resistance at either 65–85 °C or 90–125 °C temperature could be the most common criteria used to assess spore hardiness or quality. No side-by-side studies have yet been performed to compare the quality of spores generated by two or more protocols. Finally, it is not clear what alternate chemical sensitivity could possibly be used in place of commonly prescribed sensitivity to 2.5N hydrochloric acid resistance because the relevance of the latter is highly questionable.

In the context of disinfectant efficacy research for the registration of antimicrobial products, availability and use of one or more validated spore production methods is highly desirable. In addition to commonly used criteria such as heat or acid resistance of spores, predicted log reduction values, as effected by exposure to one or more sporicidal chemicals (e.g., hypochlorite, hydrogen peroxide/per-acetic, and chlorine dioxide gas), are strongly recommended as spore quality indicators.

This study was undertaken to review existing sporulation literature to down-select two additional media and to compare the quality of spores generated by the down-selected media currently used by the U.S. Environmental Protection Agency (U.S. EPA). A number of criteria were adopted for assessing spore quality (e.g., spore yield, incubation time needed for >95% sporulation, and sensitivity of the spores to physical [heat exposure] and chemical [sensitivity to bleach] treatment). The improved media for generating spores was expected to result in high spore yield, minimize the sporulation incubation period, and result in high quality spores as determined by physical and chemical criteria.

2. METHODS AND MATERIALS

2.1 Bacterial Strains, Media, and Chemicals

The bacterial strains, media, and chemicals needed for generating spores for the method are listed below:

- *B. subtilis*
- Amended Nutrient Agar, fortified with $MnSO_4$ -Difco Nutrient Agar Cat # 212000
- Synthetic Sporulation Medium composed of 5 mM KPi Buffer, 10 mM $(NH_4)SO_4$, 20 mM Na Glutamate, 100 mM MOPS, pH 7, 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 µM $MnCl_2$, 5 µM $FeCl_3$,1 µM $ZnCl_2$, 2 mM Thiamine, 50 µg/mL Tryptophan, 25 µg/mL Methionine, 100 mM Glucose, and 100 mM Malate, pH 7 (Vasantha and Freese, 1979)
- 2xSG Media composed of 1.6% Nutrient Broth or Agar, 0.05% $MgSO_4$, 0.2% KCl, 1 mM $CaNO_3$, 0.1 mM $MnCl_2$, 1 µM $FeSO_4$, and 0.1% Glucose (Leighton and Doi, 1971)
- Long Version Nutrient Agar composed of Nutrient Agar, 0.5% Beef Extract, 0.5 % NaCl, 1% Anatone (peptone), and 5 ppm $MnSO_4$. After boiling the 1 L mix, the media was cooled to 45 °C, and then filtered through a Whatman filter paper #4, pH was adjusted to 6.8 + 0.2, and $MnSO_4$ was added, before autoclaving

2.2 Methods

The needed broth media and media plates were prepared and sterilized per the prep sheet. A 10 µL aliquot of sterile media was aseptically transferred into 50 mL tubes. Each media plate was streaked from the spore stock/room temperature stab/frozen cell stock with a 10 µL loop. The plates were incubated at 36 ± 1 °C for 20 ± 4 h. Two to three single colonies from the plate were inoculated in 10 mL broth of the same media, and then they were grown for 24–48 h (depending on the growth rate in a given medium approaching the stationary phase) at 36 ± 1 °C with shaking at 175 ± 5 rpm. Dilutions were prepared from the final culture (labeled 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). The undiluted broth culture was labeled 10^{0}. An aliquot of 200 µL broth was seeded (from each undiluted or diluted set of tubes) on media plates (two plates each with 200 µL of each dilution tube). The plates were then incubated at 36 ± 1 °C. A 200 µL aliquot of broth culture was transferred to 10 mL of broth (three tubes per inoculation titer). The broth was then incubated at 36 ± 1 °C with shaking at 175 ± 5 rpm. The seeded plates and inoculated tubes were microscopically examined until 95% sporulation at an interval of 18–24 h.

The spores were harvested using the U.S. EPA Microbiology Lab method, which required adding 25 mL of cold sterile deionized water to the plates and then dislodging the

spores using an L-shaped spreader. The dislodged spores were pooled into centrifuge tubes, washed, and then centrifuged three times. The spore suspension was stored at 4 °C. The spores generated from the different media were compared for spore quality by heat and chemical resistance. For heat resistance testing, 1 mL aliquot of spore suspension (7 logs/mL) was exposed for 30 or 60 min at 65 or 85 °C. For chemical resistance testing, the inoculated carriers (~7 logs/carrier) were exposed to 3000 ppm bleach (pH-unadjusted) for 30 min. The samples were then processed using the TSM method (Tomsino et al., 2010).

3. RESULTS

3.1 Sporulation and Spore Yield

B. subtilis cells sporulated to >90% on both 2xSG Agar and Long Version Nutrient Agar within 6 days, which is significantly less time than the 12–14 days taken by the Amended Nutrient Agar media currently used by the EPA laboratory. The Table below summarizes the post-inoculation period required for >90% sporulation and the spore yield for each media tested. None of the broths tested supported sporulation as well as the agar counterpart of each. Surprisingly, as seen in the Table, no sporulation was observed when the Synthetic Sporulation media (either broth or agar) was inoculated. The sporulation progression in the broth media was significantly less (50–60%) even after 2 weeks of culture.

Table. Sporulation Progression and Spore Yield vs. Sporulation Media

Run #	Name of Media	Date Seeded	Date Harvested	Sporulation %	Days to Sporulate	Spore # Harvested
Agar						
1	Amended Nutrient Agar	6/12/2009	6/19/2009	>90	7-10 days	6.5×10^9
2	Amended Nutrient Agar	7/22/2009	8/3/209	>90	12 days	4.0×10^9
1	2xSG Agar	9/16/2009	9/24/2009	90-95	8 days	4.2×10^9
2	2xSG Agar	10/16/2009	10/22/2009	>95	6 days	3.3×10^9
3	2xSG Agar	10/29/2009	11/4/2009	>90	6 days	7.7×10^9
1	Long Version Nutrient Agar	10/8/2009	10/16/2009	>90	6 days	1.2×10^9
2	Long Version Nutrient Agar	11/9/2009	11/16/2009	>90	7 days	5.4×10^9
3	Long Version Nutrient Agar	1/15/2001	1/21/2010	>90	6 days	3.1×10^9
1	Synthetic Sporulation Agar	11/3/2009	N/A	0	Never sporulated	N/A
Broth						
1	Amended Nutrient Broth	6/12/2009	6/22/2009	99	10 days	5.1×10^8
2	Amended Nutrient Broth	7/22/2009	N/A	No Growth		
1	2xSG Broth	9/16/2009	9/28/2009	50	12 days	2.0×10^8
2	2xSG Broth	10/16/2009	10/29/2009	>60	13 days	7.6×10^7
3	2xSG Broth	10/29/2009	11/11/2009	<40	13 days	2.3×10^8
1	Long Version Nutrient Broth	10/8/2009	10/26/2009	<40	16 days	1.8×10^8
2	Long Version Nutrient Broth	10/22/2009	10/28/2009	<50	6 days	7.8×10^8
1	Synthetic Sporulation Broth	11/3/2009	N/A	10	Never sporulated	N/A

3.2 Effect of Inoculated Cell Number on Spore Yield

The number of cells (10^8 vs. 10^4), seeded in broth or on plates, did not appear to impact the spore yield because a comparable number of spores were recovered despite the cell number seeded (Figures 1–3). This result was observed for all tested media, 2xSG Agar, Amended Long Version Agar, and broth.

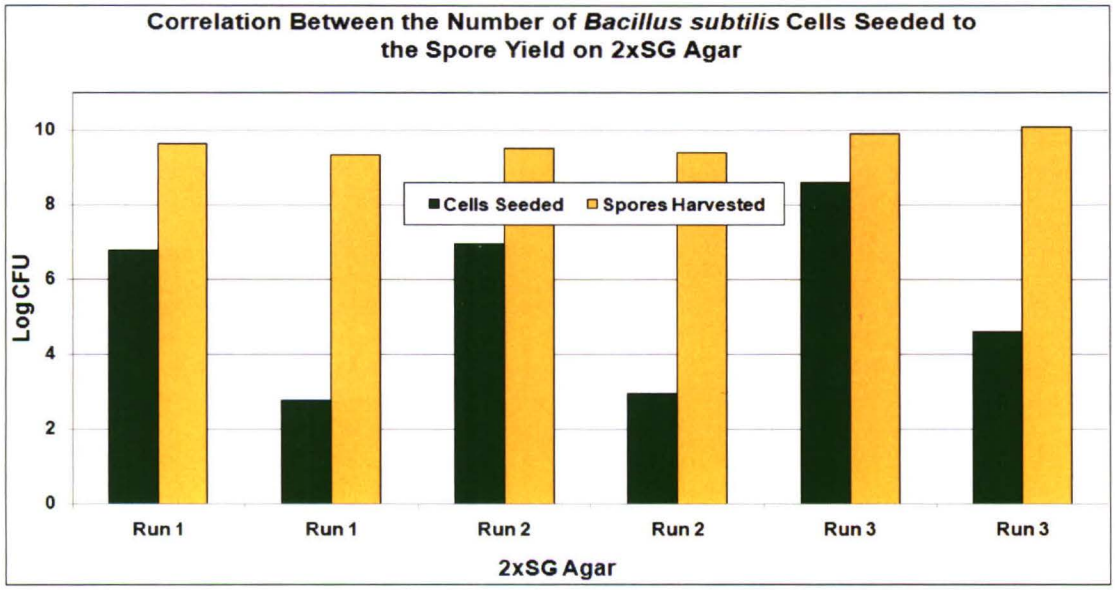

Figure 1. Correlation between cell number of inoculated *B. subtilis* and spore yield on 2xSG Agar

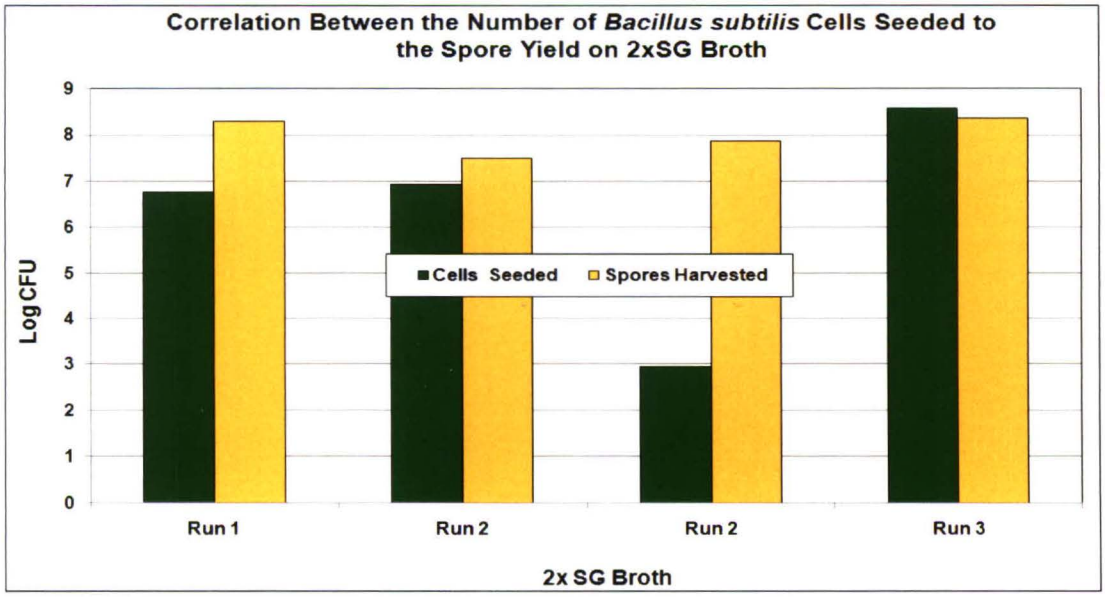

Figure 2. Correlation between cell number of inoculated *B. subtilis* and spore yield on 2xSG Broth

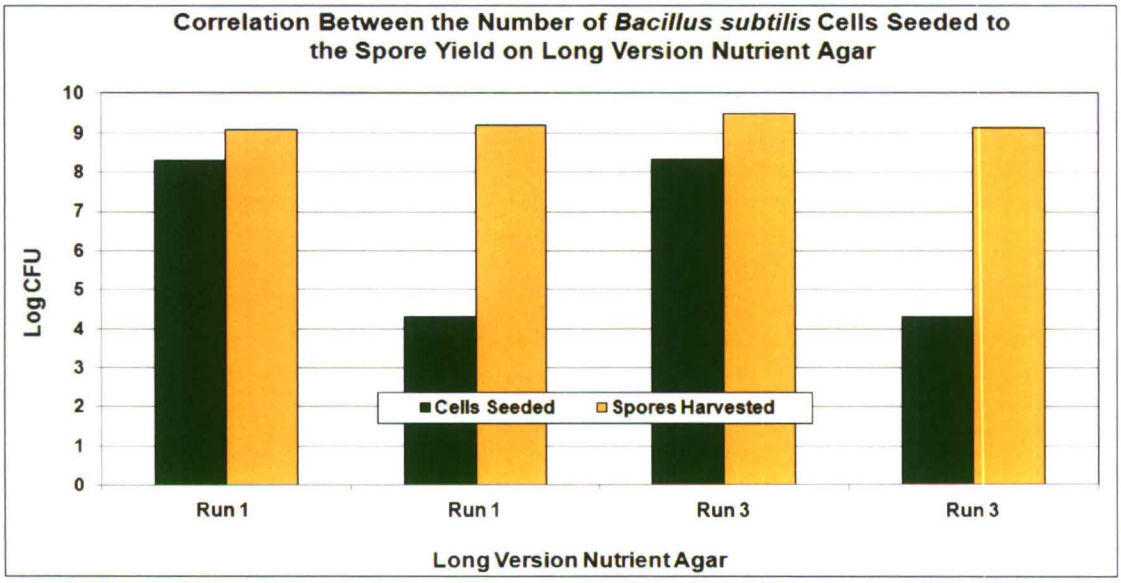

Figure 3. Correlation between cell number of inoculated *B. subtilis* and spore yield on Long Version Nutrient Agar

3.3 Spore Quality–Physical Treatment

Spore hardiness was investigated by exposure of spore suspension to 65 or 85 °C. In general, the spores harvested from the broth or plate version of 2xSG and Long Version Nutrient media, appear to be resistant to 65 and 85 °C. However, one log higher log reduction in spore viability was observed when the broth grown spores (as opposed to those grown on plates) were exposed for 30 min to 85 °C. The results from the heat treatment experiments are summarized in
Figures 4–8.

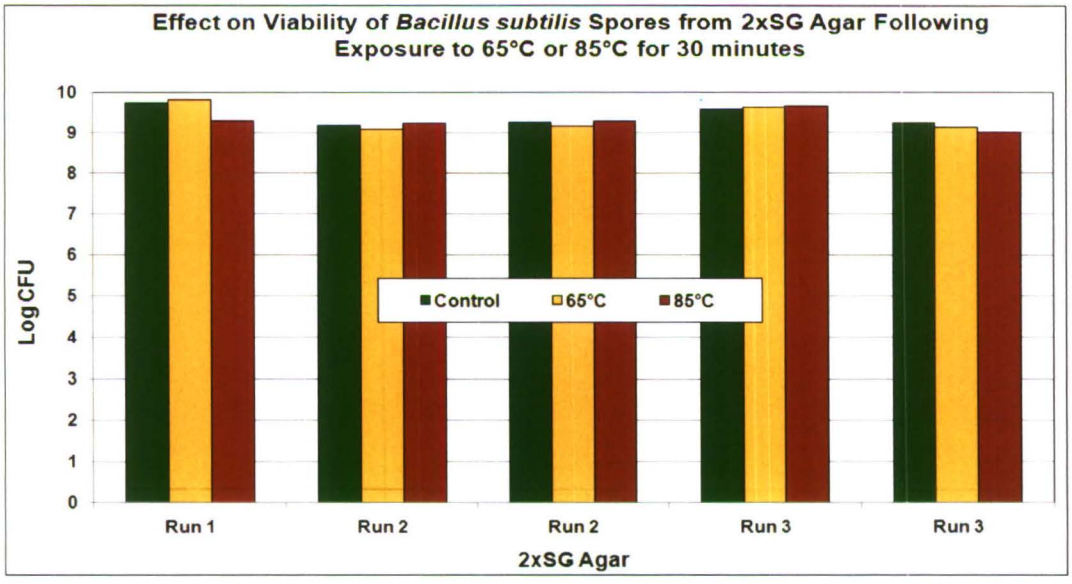

Figure 4. Viability effect of exposure on spores from 2xSG Agar to 65 or 85 °C for 30 min

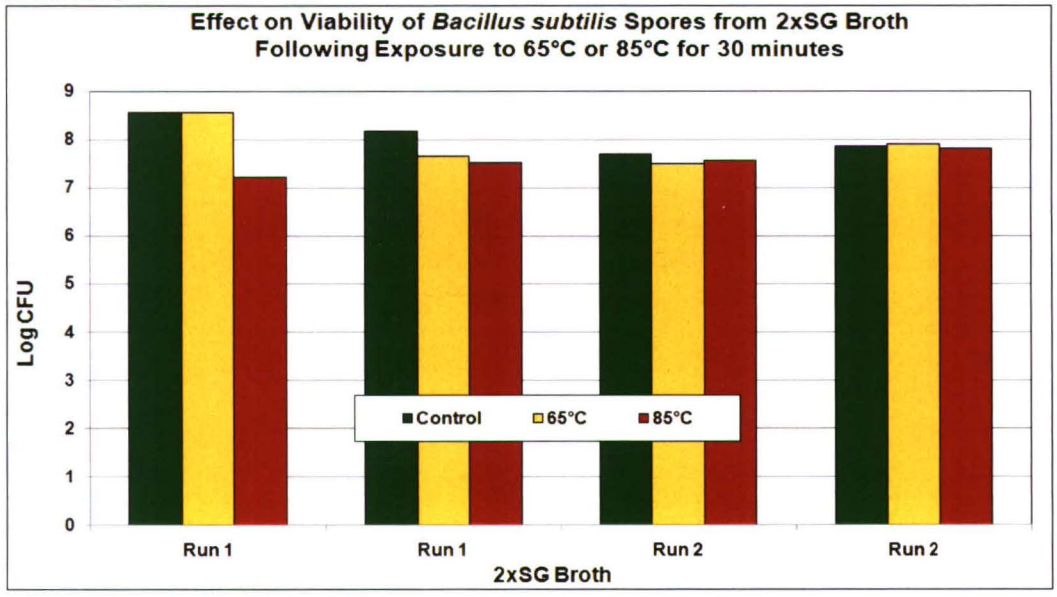

Figure 5. Viability effect of exposure on spores from 2xSG Broth to 65 or 85 °C for 30 min

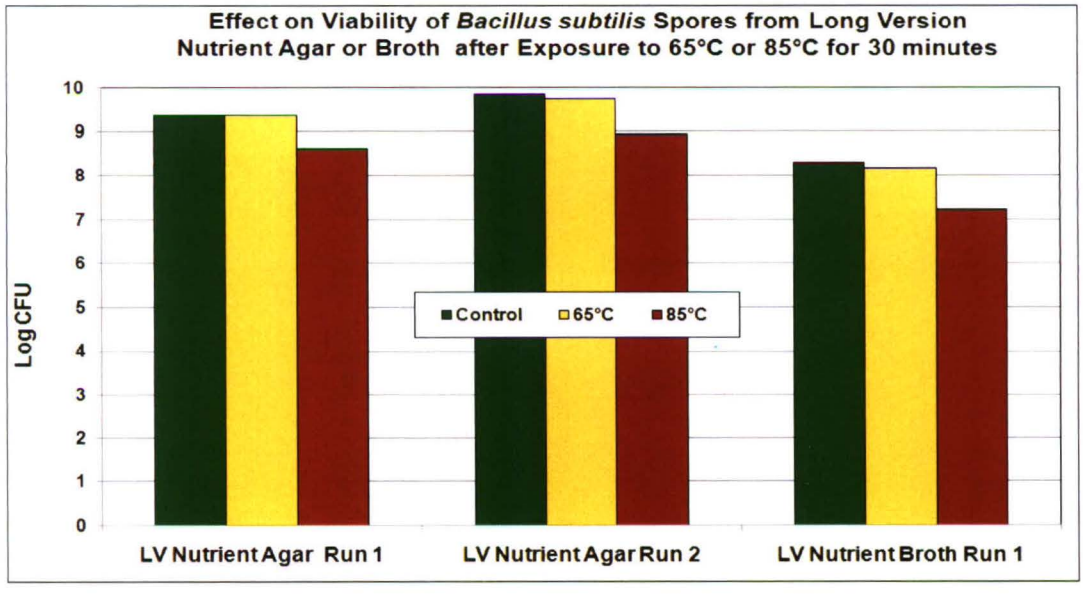

Figure 6. Viability effect of exposure on spores from Long Version Nutrient Agar or Broth to 65 or 85 °C for 30 min

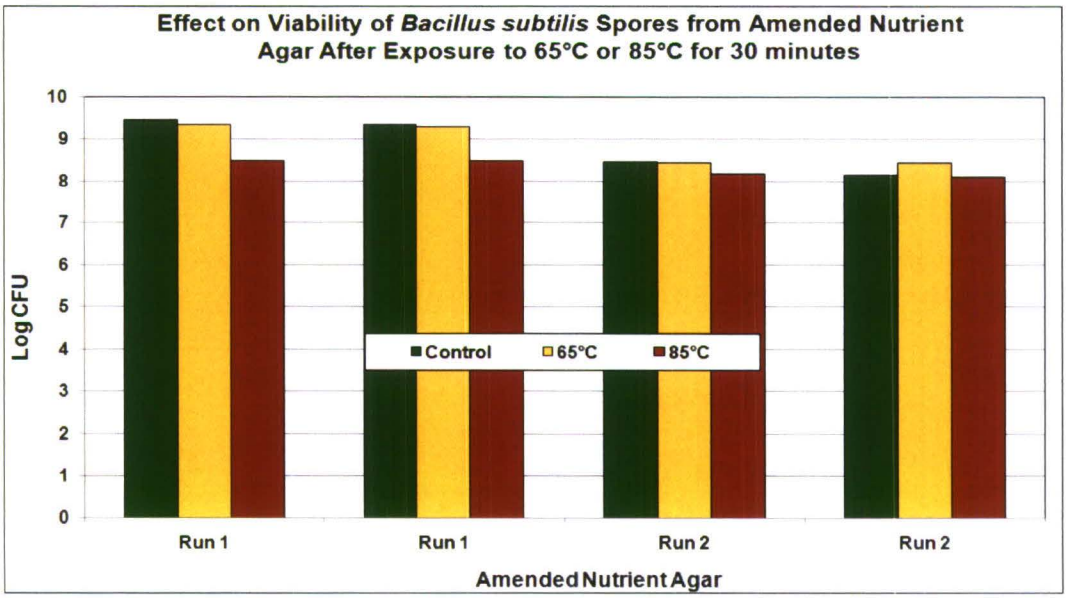

Figure 7. Viability effect of exposure on spores from Amended Nutrient Agar to 65 or 85 °C for 30 min

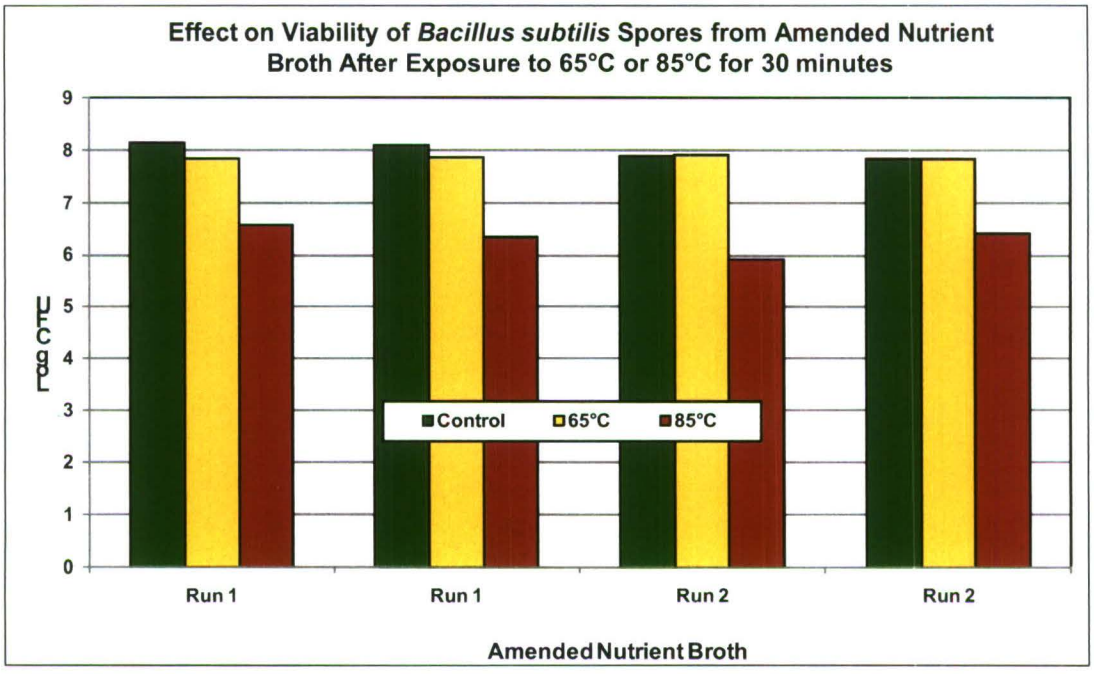

Figure 8. Viability effect of exposure on spores from Amended Nutrient Broth to 65 or 85 °C for 30 min

3.4 Spore Quality–Chemical Treatment

The spores were tested for their resistance to chemical treatment (30 min exposure to 3000 ppm pH-unadjusted bleach). The Three-Step Method was used to evaluate the relative sensitivity of spores generated by using different media. Irrespective of the media and the broth or plate version, the spores showed comparable log reduction values following intermediate bleach treatment (3000 ppm for 30 min), as seen in Figures 9 and 10. Figure 10 shows that comparable spore numbers were recovered from control glass carriers.

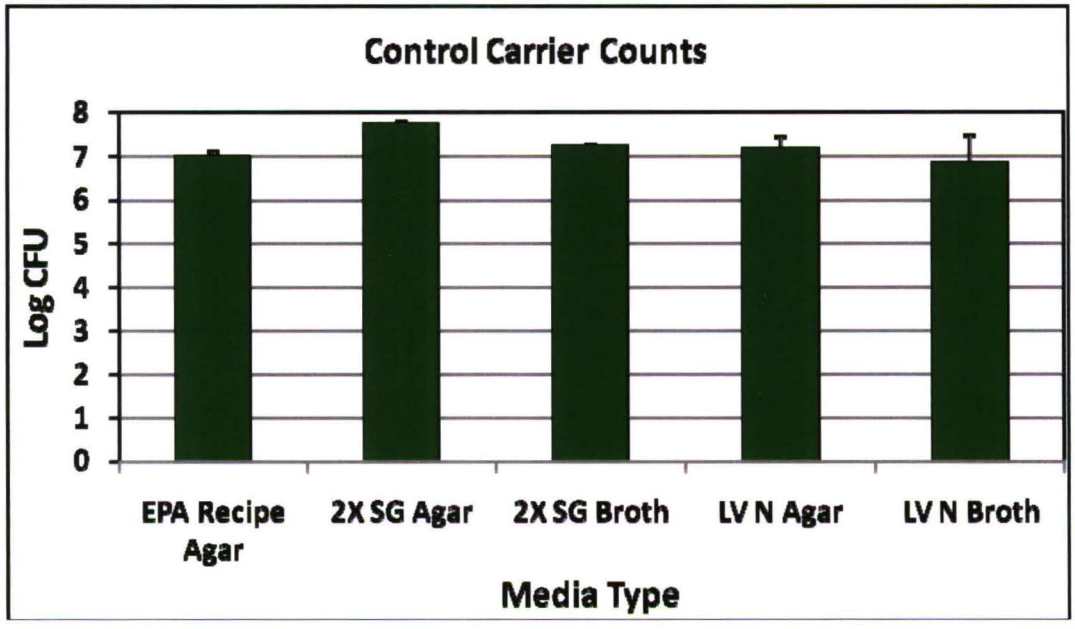

Figure 9. Control carrier counts with spores generated by different media

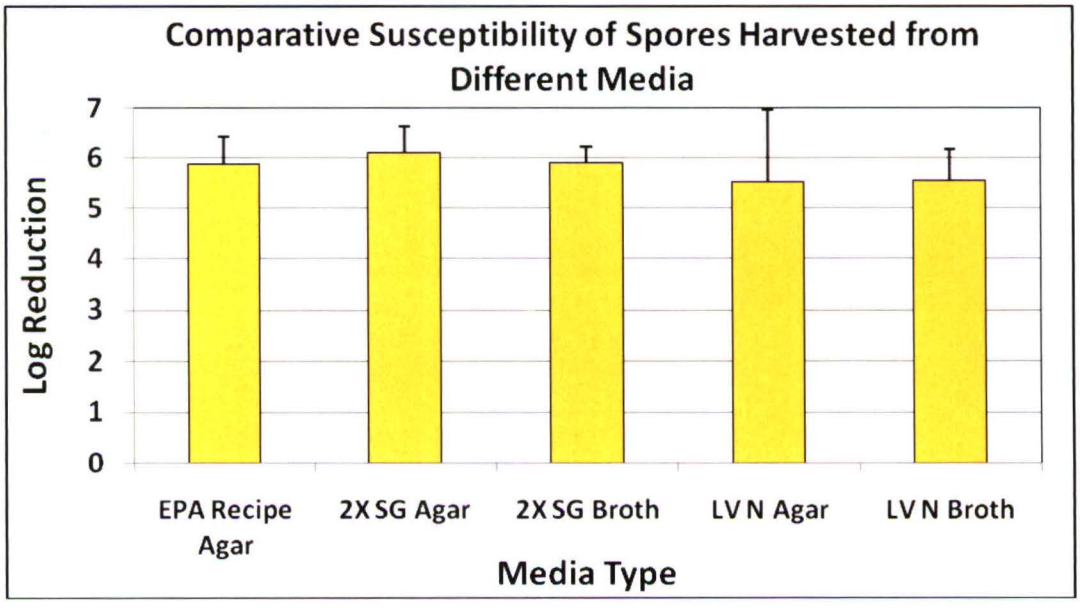

Figure 10. Log reduction in response to intermediate level of bleach treatment

4. DISCUSSION AND CONCLUSIONS

An array of broth and semi-solid agar media have been used for generating *Bacillus subtilis* spores. The media ranged from completely defined (Hutchison et al., 1974; Molin et al., 1976; Vasantha et al., 1979; Jetiyanon et al., 2008) to fully complex in composition. The goal of this study was to explore an alternate sporulation media to the one (Manganese-Amended Nutrient Agar) currently used by EPA. A few clear objectives were to maximize the spore yield, minimize the incubation period, and propose use of alternate indicators of spore hardiness (i.e., heat resistance [physical] and sensitivity to the intermediate level bleach [chemical]).

B. subtilis cells sporulated to >90% on the 2xSG and Long Version Agars within six days, which is significantly less time than the 12–14 days taken on the Amended Nutrient Agar. The spore yield from the 2xSG and Long Version Nutrient Agars was comparable (10^9 spores/mL per plate). Strangely, no sporulation was observed when cells were grown on the Synthetic Sporulation media (either broth or agar formulation). This is in contrast to the reported observation of Vasantha and Freese (1979). The sporulation progression in all types of broth media tested was incomplete and significantly lower (50–60%) even after 2 weeks of culture. This was also unexpected as sporulation of *B. subtilis* cells has been reported in the Amended Nutrient, 2xSG, and Synthetic Sporulation Broths (Vasantha et al., 1979; Leighton and Doi, 1971). This discrepancy may be due to the absence of a growth requirement specific to the strain of the *B. subtilis* used. The correlation of the initial cell number inoculated in the media and the final spore yield was also investigated in this study. Based on the results summarized in Figures 3 and 4, the number of cells seeded in broth or on plates did not appear to impact the final spore number harvested, indicating sporulation induction after a certain threshold level of growth and cell number increase were achieved.

One of the goals in this study was to explore the indicators that can be used for assessing spore quality or hardiness. Oddly, no single common set of spore hardiness indicators (e.g., spore yield, heat resistance, acid resistance, or radiation resistance) has been used within different laboratories. In this study, heat and bleach resistance were investigated for use as indicators of spore quality. In general, the spores harvested from either broth or plate versions of 2xSG and Long Version Nutrient media appear to be resistant to heat exposure at 65 and 85 °C. However, compared to the spores harvested from plates, those harvested from media broth appear to show greater susceptibility to heat exposure at 85 °C (≥1-log kill). With respect to the chemical treatment, spores showed similar susceptibility to 3000 ppm bleach, irrespective of whether they were prepared from either broth or plate version of the media. In addition to commonly used criteria such as heat or acid resistance of spores, predicted log reduction values, effected by exposure to one or more sporicidal chemicals (e.g., hypochlorite, hydrogen peroxide/pre-acetic, chlorine dioxide gas), are strongly recommended for use as spore quality indicators.

In conclusion, the present study adequately addressed the following key objectives:

- Selection and screening of sporulation media to achieve high sporulation in a shorter period of time than seen in the Three Step Method
- Selection of some relevant indicators for assessing spore quality and hardiness

Because sporulation in the broth versions of the media selected in this study were significantly <90%, additional work is recommended for testing other broth media, such as G media (Stewart, et al. 1953). Sporulation progression was within 7 days in the 2xSG media; therefore, further work should be performed on other *Bacillus* species, including *B. anthracis*. As no growth was achieved on fully synthetic sporulation media, additional recipes of chemically-defined media should be tested. Further work in terms of multi-lab validation is also recommended on implementation of thermal sensitivity and bleach resistance instead of acid resistance as spore quality indicators. Finally, selection and application of genetic/biochemical markers are also recommended for spore quality or their hardiness.

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