AN IMPROVED METHOD FOR THE PRODUCTION OF *Bacillus subtilis* var. *niger* SPORES FOR USE AS A SIMULANT FOR BIOLOGICAL WARFARE AGENTS- QUALITY ANALYSIS

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ABSTRACT

Standards for proliferation of Biological Warfare (BW) agent simulants for use in development of detection and identification equipment are essential. Lack of standardized protocols for growth, processing, and product characterization will likely lead to variances in growth parameters and could induce changes in simulant characteristics that may affect instruments being developed. We have evaluated thirteen media purported to grow spores against several criteria, including growth time, ease of processing, reproducibility, and component definition. The goal is to have a chemically defined medium that will produce whole bacillus spores in the least amount of time. Three media were selected for further testing. Spores produced from each medium were tested for viability counts, purity, particle size, identity, and appearance under electron microscope. The spores exhibited similar viability counts per dry weight, do not contain extraneous organisms, and are virtually free of debris. Particle size is comparable when grown on each medium and spores are easily aerosolized and measured by an Aerodynamic Particle Sizer (APS). Identity testing using infrared spectroscopy and PCR indicate positive identity with no major interference factors. Data indicates suitability for use in BW agent detector development.

INTRODUCTION

Bioterrorism is a real and deadly threat in today's society. The proven use of microorganisms as weapons has lead to a much greater awareness of a constant, until recently, solely Joint Forces mission; Nuclear, Biological and Chemical (NBC) Defense Preparedness. The deadliest method of anthrax infection is through inhalation of aerosolized spores[1] and the most useful way to test for this type of threat is through the use of simulant non-pathogenic spores. *Bacillus subtilis* var. *niger* (formerly *Bacillus globigii*, a.k.a. BG) is the simulant of choice used to model anthrax. It is essential that the R & D community have a quality, reproducible, standardized simulant spore organism at their disposal in order to establish efficacy of emerging and existing protection, detection and decontamination technologies. The intent of this project is to standardize the production of pure and clean BG spores by defining required materials and methods. Specifically, this was accomplished by the identification of media formulations, reduction of growth time, and efficiency of harvesting and processing. Chemically defined media are

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advantageous in that components have fixed content in contrast with components derived from animal products. Liquid media are advantageous for growing microorganisms on a large scale. Previous media studies show that NSM was the best spore-inducing formula when compared to twelve other liquid and solid media. CAD was one of the more successful liquid formulations, therefore was included with a minor ingredient substitution (casein product). Rounding out the media formulations, a completely chemically defined liquid media, CDSM, was also evaluated.

MATERIALS AND METHODS

Growth trials comparing three different media formulations, (Table 1); Nutrient Sporulation Media (NSM) a solid growth medium, Casein Acid Digest (CAD) a liquid medium adapted from Military Specification MIL-S-50003A (CmlC)[2], and Chemically Defined Sporulation Medium (CDSM) as described by Hageman et. al.[3]. Media were prepared and checked for contamination prior to inoculation. CAD and NSM cultures were incubated under ambient air 30+/-3 °C; the CDSM cultures were incubated under ambient air at 37+/-3 °C . Liquid media (CAD and CDSM) were aerated at 250+/-10 rpm. Two strains of *Bacillus subtilis* var. *niger* were used; ATCC 9372 and in house frozen stock from Dugway Bioferm lot 10-88, the standard military BG. NSM and CAD were inoculated directly from freezer seed stock, prepared by growing in Nutrient broth to OD 600 nm of 0.9 and adding 20% vol/vol of glycerol. CDSM seed was inoculated using a single colony incubated at 37+/-3 °C, under ambient air, 250 +/-10 rpm for 18-48 hours.

Percent sporulation was determined daily by visual acuity count with Schaeffer-Fulton spore stain (5% malachite green)[4]. Several samples were observed under phase contrast microscopy for refractile body production. Samples were harvested at 80% or greater sporulation with Schaeffer-Fulton spore stain, or when the test period of 14 days was complete. Liquid media was harvested by centrifugation at 10,000g for 30 minutes. Solid media was harvested by scraping and rinsing the surface with sterile distilled-deionized water (dDI). Samples were heat-treated in 70 °C water bath for 30 minutes with occasional shaking. Samples were centrifuged at 10,000g for 30 minutes and subsequently washed three times in dDI. The final pellet was resuspended in dDI, aliquoted and lyophilized. Finished vials were stored at –30 °C. Percent viable spore counts (%VS) were determined by dilution in Tryptose broth and plating onto Tryptose agar (BD-Difco, Cockeysville, MD). Percent variation and contamination was determined by counting the number of variant and contaminant colonies present of the viable spore dilution plates[5]. For electron microscopic analysis samples were affixed to conductive carbon tape (SPI supplies, West Chester, PA) on aluminum mounts and examined with no further sample preparation. Scanning Electron Microscopy (SEM) was done using a JEOL 6300F Field Emission SEM (JEOL, Tokyo) calibrated against a 2160 line-grating replica to within 1%. The measurements were taken manually using Vision software (JEOL USA, Peabody, MA), operating in point-to-point measurement mode because particle aggregation and agglomeration precluded reliable use of fully automated particle sizing software. Spores were measured across the shortest distance (minor dimension) and longest distance (major dimension). Only spores that were entirely visible and unobstructed by other materials or spores were measured. Data handling and charting was done using Microsoft Excel. Energy Dispersive X-Ray Spectroscopy was done at 15KeV using a Noran Voyager spectrometer with light element detector. The TSI Model 3433 Small-Scale Powder Disperse (SSPD) was used to aerosolize and disperse BG preps. The SSPD aspirates single particles and agglomerates of particles up to 50 microns, de-agglomerates and presents the aerosolized samples to the APS. The TSI model 3310 Aerodynamic Particle Sizer (APS) uses time-of-flight calculations to measure the aerodynamic particle size of samples. Particles in the airstream are separated by size based on differences in individual particle size and inertia and measured as time-of-flight across a split laser beam and photo detector.

IR spectra were generated using the Travel*IR* Fourier Transform Infrared (FT-IR) spectrometer from Sens*IR* Technologies. Dried samples were placed directly on the instrument for analysis. The Travel*IR* FT-IR device uses multiple software systems for detecting and analyzing infrared spectra. The primary database search tool, "QualID", automatically matches the test spectrum with reference spectra from a database library and reports results based on set parameters. By subsequently employing the "Grams" program one can further evaluate samples by subtracting known interferents from desired spectra. This method was used by scanning the Dugway stock material and subsequently subtracting the spectrum for silica from the Dugway spectrum. This final spectrum was added to the instrument's library and used as a basis for comparison of the test material.

PCR testing was completed using the RAPID LightCycler AP0097. A loop full of each dried sample was suspended into 1 ml Molecular Biology Grade water, vortexed well then diluted 1:10 in same and vortexed. Resuspended samples were prepared and run using standard LightCycler methodology.

RESULTS

Of the three media evaluated, NSM is the best for induction of spores by *B. subtilis* var. *niger*, BG, based strictly on growth time, percent sporulation and bulk of material produced on average (Figure 1). The overall gram yield of spores per media type shows that NSM produces the greatest spore mass, averaging about 1 gram of dry spores per liter. Furthermore, BG Dugway lot 10-88 produced more fully formed spores in a shorter time, on each of the media as compared to the ATCC strain 9372.

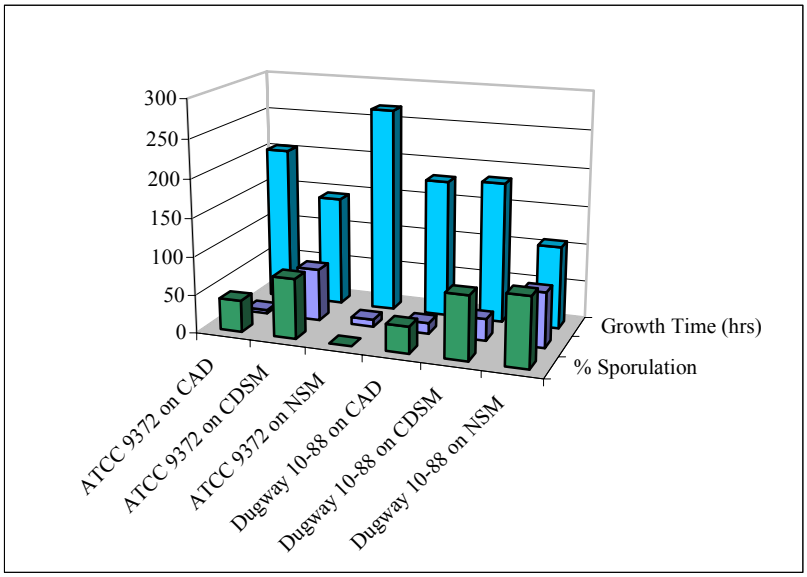

Figure 1. Summary of Percent Sporulation of Bacillus subtilis var niger (BG) Grown on Various Media.

Enumeration of dried spore mass for viable spores per gram (Figure 2), indicates that both Dugway and ATCC strains on NSM, as well as the Dugway strain on CAD, provide the required 10^{12} colony forming units per gram. The ATCC strain was not able to produce a sufficient number of viable spores on CAD and CDSM to meet our criteria. The Dugway strain on CDSM yielded inadequate results for one of the growths and required further testing.

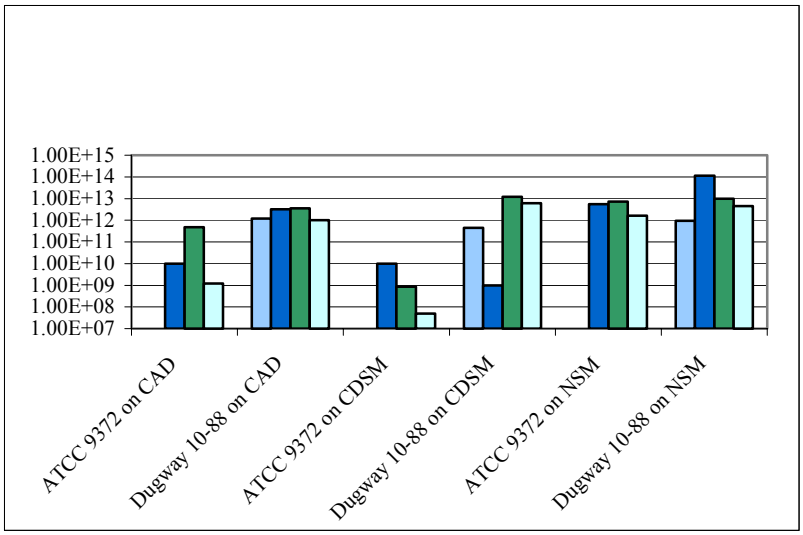

Figure 2: Yield of Viable Spores per gram (VS/g) for two strains of Bacillus subtilis var. niger on Various Media

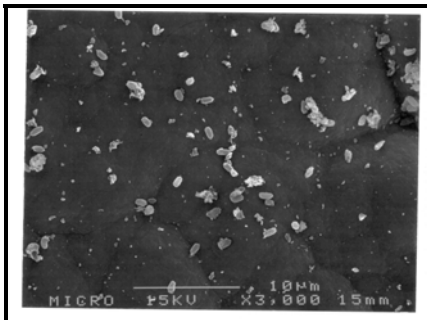

Figure 3A: Electron micrograph of *Bacillus subtilis* var. *niger* from Dugway stock

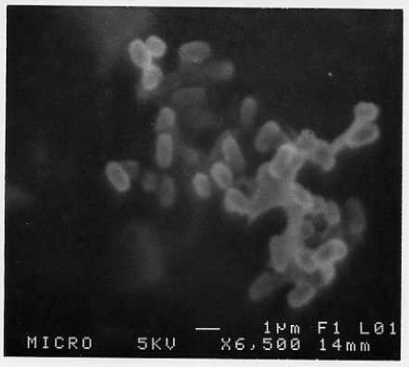

Figure 3B: Electron micrograph of *Bacillus subtilis* var. *niger* spores on CAD medium

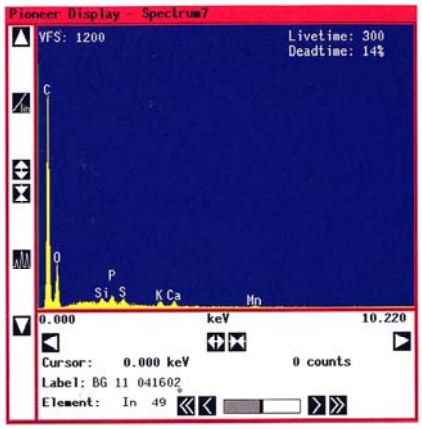

Figure 3C: Spectral Analysis of *Bacillus subtilis* var. *niger* spores on CAD medium

In figure 3, visualization of BG spores from an Old Dugway (A) preparation under electron microscopy (3,000X) shows the presence of a large amount of debris in the field. Visualization of BG spores grown on CAD medium under electron microscopy (6,500X) shows fully formed, non-agglomerated spores (B). The spectral analysis indicates no residual interferents are present in the CAD spore preparation (C).

API measurement (data not shown) of the median diameter of spores grown on the three media show comparable results to Dugway stock material, at approximately one micron, when measuring 80 to 90,000 particles. Infrared spectral analysis shows that both of the BG strains grown on each of the three media were detected in the top ten hits as compared to the Dugway Bioferm Lot 10-88 (after subtraction of silica).

Figure 4 shows a positive identity and visual comparison of one of the tested preparations with the Dugway stock material. LightCycler Analysis (Figure 5) of spores grown on NSM and CAD media gave positive identity indicating that growth media, parameters or processing method had no adverse effect on sample quality nor did these contribute interfering factors.

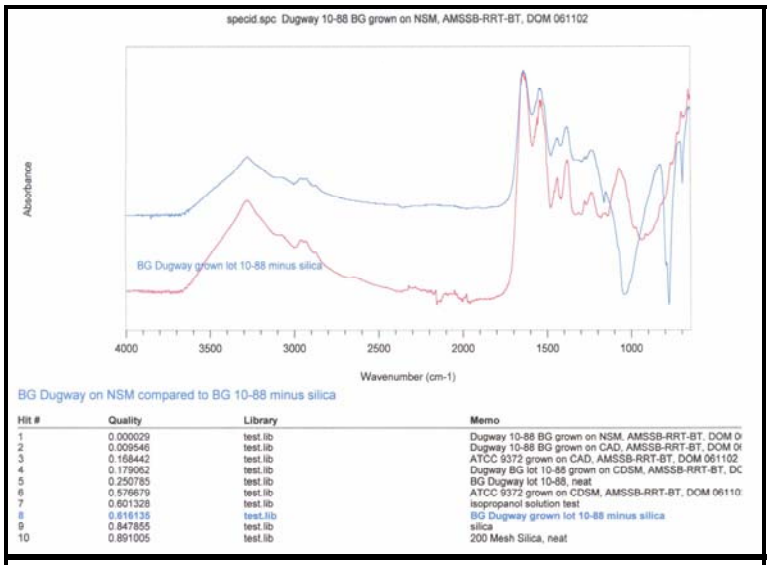

Figure 4: TravelIR Infrared spectral analysis of BG grown on NSM medium (bottom) as compared to the spectra from Dugway stocks (top) after subtraction of

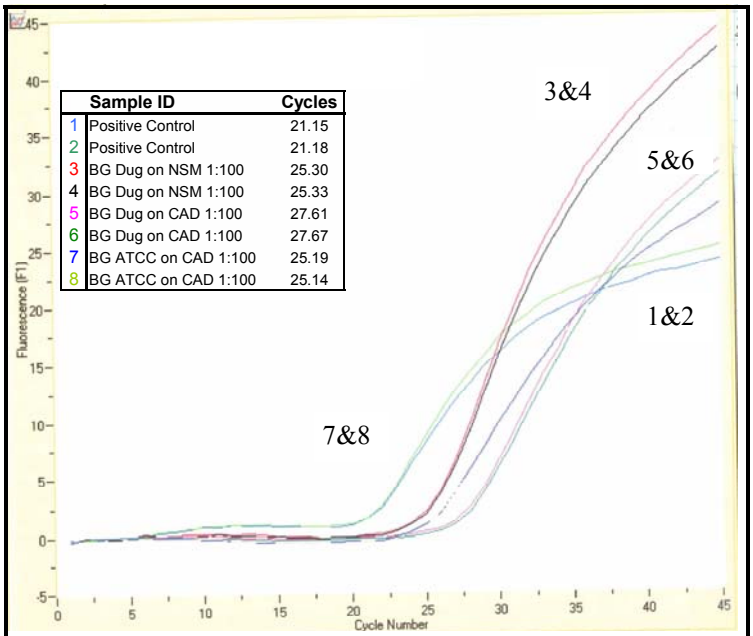

Figure 5: PCR analysis using RAPID LightCycler . Test BG spores grown on NSM and CAD media show comparative identity to positive control (lanes 1 and 2).

DISCUSSION

NSM, while a better spore producer, is less advantageous than the liquid CAD and CDSM formulations. Liquid media are easier to work with on a larger scale and adaptable for use in fermentation vessels. A chemically defined medium is preferable to a medium containing animal

products due to the simple fact that ingredients do not vary in elemental and chemical compound quantity or content. CDSM was designed for growth, sporulation, and production of extracellular proteases to better allow metabolic studies of nutritional auxotrophs of *Bacillus subtilis*. Sporulation was determined by examining a cell suspension under phase contrast microscopy and determining the number of retractile bodies present.[3] Vegetative cells do show refractility upon initiation of sporulation, however, since we require fully formed and released spores, refractility is not a sufficient measure for our testing. Therefore, percent sporulation was determined by released spores seen using the Schaeffer-Fulton spore stain method. Although CDSM induces endospores in a relatively short period of time when compared to the CAD and NSM, for at least one of the growth trials the majority of endospored cells did not completely sporulate by our criteria before lysis. Optimization and analysis of CDSM relative to seed culture and inoculum will continue. Furthermore, investigations are being conducted with variations of the CDSM to elucidate modifications to achieve complete sporulation by our criteria.

Our size criterion of 1-5 microns is based on the fact that particles remain effectively aerosolized in this range[7]. Particles larger than five microns are not effectively suspended in the air for long periods of time and are not efficiently inhaled. Since small particles accelerate more quickly than large particles, changes in particle velocity are proportional to their size. Particles are measured in an air-stream and separated by flight characteristics; therefore, the sizes reported are the aerodynamic particle size. The measurement of particle size is independent of particle shape but is influenced by shape and surface characteristics. Electron microscopy, elemental analysis, Infrared spectroscopy and PCR indicate that no residual media components or cellular debris is present in the spore preparations to act as interferents.

Spores originating from the Dugway material then proliferated on the tested media under stated conditions are acceptable for use as a standardized simulant. Spores from the ATCC strain were not effectively induced to meet our criteria on CAD and CDSM, however, on NSM the levels were acceptable but only after an extended growth period. Particle size and spectral analysis in conjunction with; yield of viable spores, percent sporulation, dry mass, growth time, and lack of atypical colonies support the validity of these methods.

CONCLUSIONS

Each of the three media tested are capable of inducing spore production by the each of the tested strains of bacteria *Bacillus subtilis* var. *niger*. Harvesting, handling and processing of spores by the described method provided clean, pure and unadulterated samples as proven through testing with traditional bacteriologic techniques, electron microscopy, aerosolization, infrared spectroscopy and polymerase chain reaction. This battery of assays demonstrates that BG spores grown herein are suitable for use as a gold standard simulant for sensitive technologies currently in use and under development. NSM medium produced a higher yield of spores in a short time but posed logistical issues due to the solidity of the medium. CAD medium produced a somewhat smaller yield in a reasonable time but proved to be less predictable than NSM overall. CDSM delivered varied results with lower yield than both NSM and CAD. However, CDSM (being chemically defined) holds promise and work with this medium is continuing.

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