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PRODUCTION OF *BACILLUS* SPORES AS A SIMULANT FOR BIOLOGICAL WARFARE AGENTS

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RESEARCH AND TECHNOLOGY DIRECTORATE

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14. 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. Thirteen media purported were evaluated 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 (two liquids and one solid) were selected and tested further. Although the solid medium produced the best results, it is more advantageous to use a liquid medium. There is a liquid medium that is chemically defined that produced refractile bodies in twelve hours. Ingredient variations were tested to determine impact on sporulation levels. The spores are virtually free of all media components and debris. Particle sizing of the spores proliferated on all three of the media indicate that they are suitable for use in BW agent detector development.

15. SUBJECT TERMS

BG	*Bacillus subtilis* var. *niger*	Spores
MIL SPEC	Military specification	*Bacillus* spores
CDSM	Chemically defined sporulation medium	Simulants

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PREFACE

The work described in the report was authorized under Project No. CDP2019, the Technical Base Project, Bacterial Simulant Production for Biological Warfare (BW) agent testing. The work was started in September 2002 and completed in September 2003.

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PRODUCTION OF *BACILLUS* SPORES AS A SIMULANT FOR BIOLOGICAL WARFARE AGENTS

1. INTRODUCTION

The proven use of microorganisms as weapons has lead to a much greater awareness of a constant Joint Forces mission; Nuclear, Biological and Chemical (NBC) Defense Preparedness. With recent world events, specifically the use of *bacillus* spores as weapons of mass destruction, an increased focus on biological defense and technology necessitates the need for increased amounts of simulant organisms used in research and development of new detection, identification and decontamination technologies. It is, therefore, essential that the Research and Development (R&D) community have a quality, reproducible, standardized simulant spore organism at their disposal to establish efficacy of emerging and existing decontamination, detection and protection technologies.

The deadliest method of anthrax infection is through inhalation of aerosolized spores; a practical 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 historically the military community's simulant of choice used to model *Bacillus anthracis*, the causative agent of anthrax. The intent of this project is to standardize the production of pure BG spores by defining required materials and methods. Specifically; the definition of media formulations, reduction of growth time, and efficiency of harvesting and processing. No current standards exist for the growth and processing of simulant biological organisms. During a search for spore inducing media formulations, a Military Specification (MIL SPEC) for BG was uncovered; written in 1961 it included antiquated media formulations and outdated methods. It was replaced by an Edgewood Area specific MIL SPEC in 1984, which was also antiquated, and subsequently cancelled "without replacement" in 1995. The antiquated MIL SPEC was based on a procedure for media preparation that is no longer practiced; namely, preparing an acid-digested casein. The remaining media components were then added at a percentage of the amount of animal protein (casein) digested; this likely caused lot-to-lot variation.

Revisions to the MIL SPEC (MIL-S-5003EA) were developed concurrently with testing. Additionally, more stringent QA/QC test methods were identified and practiced under GLP guidelines, to verify the content and purity of media and processing components, particle size, purity of sample, and to assess aerosolization capability.

Chemically defined media are advantageous in that components have fixed content in contrast with components derived from animal products whose components may tend to vary from lot to lot. Liquid media is advantageous for growing microorganisms on a large scale however; previous media studies show that solid media is the best spore-inducing formula as compared to twelve other liquid and solid

media[8]. Casein digest (CD), adapted from the MIL SPEC[7], was one of the more successful liquid formulations, and therefore was included with a minor ingredient substitution. A chemically defined liquid medium that caused stimulation of BG proteins indicative of sporulation within twelve hours was discovered during a literature search. The original intended use for this liquid chemically defined medium was not for large-scale production of fully formed spores, however, because this medium is liquid and defined; it is necessary that it be optimized. Investigation as to the method of inoculation and growth time of the seed inoculum, subsequent large volume inoculation, processing methods, and bacterial strain specificity are ongoing.

2. MATERIAL AND METHODS

2.1 Starting Materials - Bacterial Strains.

2.1.1 American Type Culture Collection (ATCC) Strain.

The ATCC 9372, *Bacillus subtilis* var. *niger* was deposited by N. R. Smith et.al and subsequently reclassified as *Bacillus atrophaeus*. The history of the strain is traced to the depositor Bacon Labs (*Bacillus globigii*) red strain, C.R. Philips (Camp Detrick).

2.1.2 Dugway Proving Ground (DPG), BioFerm Lot 10-88 Strain.

Propagated from Dugway Proving Ground by Bioferm, Lot 10-88, as described in the Final Report of Fermentation and Maintenance of Simulant Stocks, L. D. Larsen and B. G. Harper[2].

2.2 Media Evaluated.

2.2.1 Nutrient Sporulation Medium (NSM)[9].

The NSM is a solid media; partially chemically defined it is considered a minimal medium.

2.2.2 Casein Digest (CD)[7] Medium.

CD is a liquid media adapted from the MIL SPEC, partially chemically defined. Amount of ingredients are based on percentage of an acid digest of casein. This procedure is no longer routinely conducted in the lab therefore the amounts are based on calculated numbers from the percentage of digestion listed on the manufacturer's label for pancreatic digest of casein, or Casitone (BD-Difco, Cockeysville, MD).

2.2.3 Chemically Defined Sporulation Medium[6] (CDSM).

CDSM is a liquid media as described by Hageman, et al. It is a completely chemically defined medium specifically designed for sporulation of a particular strain of *Bacillus subtilis* (strain number 168).

2.3 Percent Viable Spore Count (%VS).

The number of viable spores per weight was determined by dilution in Tryptose broth (BD-Difco, Cockeysville, MD), prepared at 10% strength, and plating onto Tryptose agar (BD-Difco, Cockeysville, MD). Percent variation and contamination were determined by counting the number of variant and contaminant colonies present of the viable spore dilution plates[3].

2.4 Percent Atypical Colony Count (%ACC).

The presence and number of atypical colonies were determined by visual inspection of the Tryptose plates used in the %VS. The number of atypical and/or abnormal colonies was counted and a percentage was determined based on the overall number of colonies on the plate and the dilution factor[3].

2.5 Percent Sporulation.

The total amount of sporulation in individual growth batches was determined by visual acuity under light microscopy (1000x) using the Schaffer-Fulton Spore Stain Method[4] and staining with a 5% solution of Malachite Green.

2.6 Aerosolization of BG Spore Preparations.

The TSI Model 3433 Small-Scale Powder Disperser (SSPD) was used to aerosolize and disperse BG preparations. The SSPD asperates single particles and agglomerates of particles up to 50 microns, de-agglomerates and presents the aerosolized samples to the Aerodynamic Particle Sizer (APS). The TSI model 3310 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.

2.7 Spectroscopic Analysis.

Fourier-Transferred Infrared Spectroscopy (FT-IR) was used to gather spectroscopic data on spores grown in each of the three media. The Travel*IR* device, manufactured by Sens*IR* Technologies of Danbury CT, is used to identify unknown solids, powders, pastes, gels, and liquids through infrared spectroscopy by using a diamond crystal embedded in a stainless steel disc.

2.8 Proliferation and Processing.

Growth Parameters.

Growth trials comparing the three selected media, listed in 2.2 above (NSM, CD, and CDSM) were conducted. The Media were prepared and checked for contamination prior to inoculation. Two strains of *Bacillus subtilis* var. *niger* were used; ATCC number 9372 and Dugway Bioferm lot 10-88, also known as military BG.

NSM and CD were inoculated directly from freezer seed stock, CDSM seed was typically inoculated using a single colony incubated at 37+/-3 °C, under ambient air, 250 +/-10 rpm for 18-48 hours, however, freezer seed stock is acceptable for use. Percent sporulation was determined daily by visual acuity count with Schaeffer-Fulton spore stain, 5% Malachite Green[4]. Samples were also observed under phase contrast microscopy for refractile body production; the spore stain method provides a more clear visualization of fully formed spores whereas the phase contrast will show both fully formed spores and partially formed endospores as 'spores'; bright points of light in the field of view. There is no clear differentiation between fully formed spores and partially formed endospores.

2.9 Growth and Sporulation Determination for Each Media Evaluated.

2.9.1 NSM.

NSM plates, one liter averaging 15-18 large 100 x 150 mm plates, were inoculated with 0.1 ml freezer stock of BG, prepared by growing in Nutrient broth to OD of 0.9 at 600 nanometers (nm) and adding 20% vol/vol of glycerol, and incubated at 30 +/-3 °C in a LabLine microprocessor carbon dioxide incubator. Plates were checked at approximately 24-hour intervals. Percent sporulation was determined by taking a representative sample from one plate per strain and visualizing it under 1000x light microscopy using the Schaeffer-Fulton spore stain method. Upon visual determination that the number of spores was at least 80 percent to that of vegetative material, the plates of that strain were harvested

2.9.2 CD.

One liter of CD was inoculated with one ml freezer stock of BG prepared by growing in Nutrient broth to OD of 0.9 at 600 nanometers (nm) and adding 20% vol/vol of glycerol, and incubated at 30 +/-3 °C under ambient air at 250 rpm in a New Brunswick model C25 floor shaker incubator. Samples were drawn and checked at approximately 24-hour intervals. Percent sporulation was determined by taking a representative sample from the center of the volume of liquid per strain and visualizing it under 100x light microscopy using the Schaeffer-Fulton spore stain method. Upon visual determination that the number of spores was at least 80 percent to that of vegetative material, the strain was harvested.

2.9.3 CDSM.

A seed inoculum was prepared prior to inoculation of the liter volume by taking a young (less than 3 days growth) isolated colony of BG spores from a plate and transferring it to the small volume of seed inoculum. The seed inoculum was grown at 37 +/-2 °C under ambient air at 250 rpm in a New Brunswick model C25 shaker incubator for a period of 18-48 hours until such a time as the seed was more than 80 percent sporulated. The seed was then aseptically transferred to the larger volume and grown at 37 +/-2 °C under ambient air at 250 rpm in a New Brunswick model C25 shaker incubator. Percent sporulation was determined by taking a representative sample from the center of the volume of liquid per strain and visualizing it under 100x light microscopy using the Schaeffer-Fulton spore stain method. Upon visual determination that the number of spores was at least 80 percent to that of vegetative material, the strain was harvested.

2.10 Harvesting.

Samples were harvested at a minimum of 80% sporulation as determined through visual acuity using the Schaeffer-Fulton spore stain method, or when the test period of 14 days was complete. Liquid media was harvested by centrifugation at 10,000 rotations per minute (rpm) 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 into 3-5 ml samples and lyophilized. Finished vials were stored at minus 30 °C.

3. RESULTS AND DISCUSSION

3.1 Media Formulations and Adjustments.

Of the three media evaluated, NSM performed the best for induction of spores by BG based strictly on growth time and percent sporulation as an average of three growth trials as shown in 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, free of vegetative outer coat, in a shorter time on each of the media as compared to ATCC strain 9372.

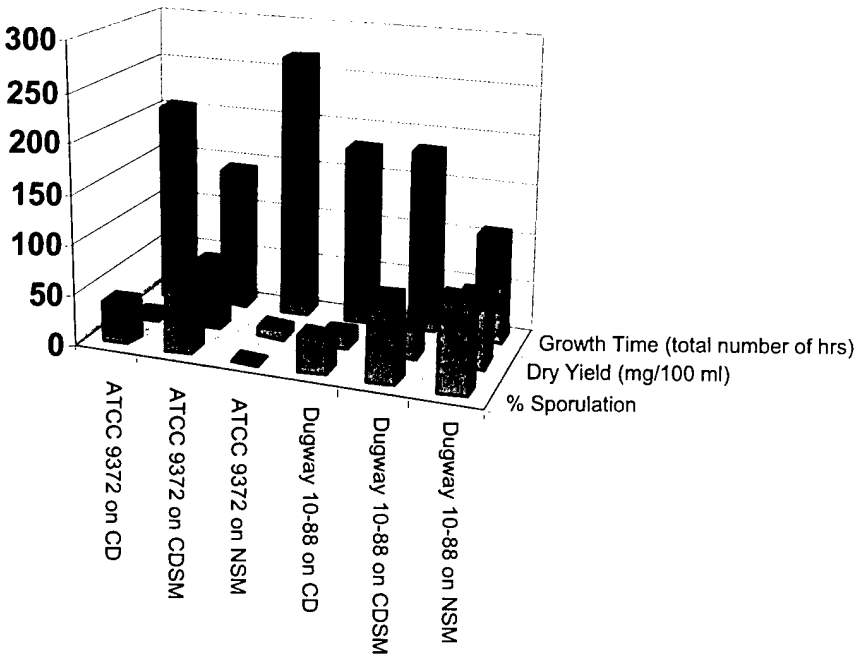

Figure 1. Comparison of Percent Sporulation, growth time and yield in milligrams per 100 milliliters, of two strains of *Bacillus subtilis* var. *niger (*ATCC 9372 and Dugway BG) Grown on Various Media

As indicated in Figure 1, the Dugway strain (DPG 10-88) displayed a shorter growth time, larger dry yield and higher percent sporulation than the ATCC strain, 9372. Figure 2, compares the yield of Viable spores per gram (%VS/g) of both strains; DPG 10-88 and ATCC 9372, by methods described in MIL-S-50003(EA)[7] and the 1965 "Purchase Description for *Bacillus* subtilis var. niger, dry sized"[3]. The results indicate that spores grown on NSM had the highest (reproducible) overall yield of spores.

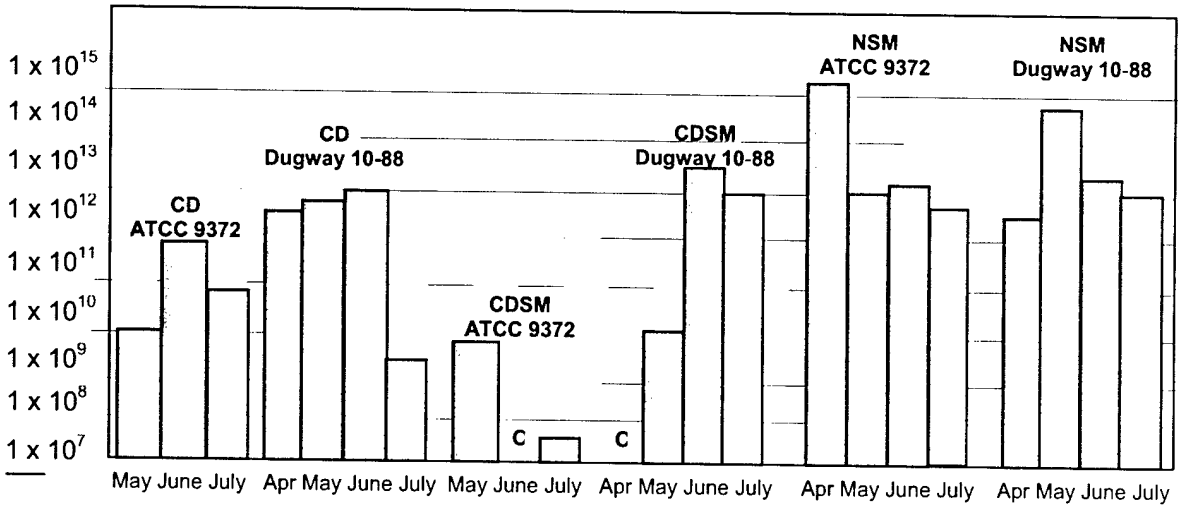

Figure 2. Comparison of Viable Spores per gram (%VS/g) yield, by date, for two strains of *Bacillus subtilis* var. *niger* on three media (CD, CDSM and NSM)

3.1.1 NSM.

While this solid media is a better spore producer, it is less advantageous than the liquid CD and CDSM formulations. Preparation and harvesting/processing time was markedly increased (at least four-fold) for NSM when compared to that of liquid media. As a partially defined medium, this leaves way for variances as a result of having animal protein as the primary nitrogen source. Enumeration of dried spore mass for viable spores per gram (Figure 2) indicates that both Dugway and ATCC strains on NSM provide the acceptable 10^{12} colony forming units per gram.

3.1.2 CD.

This partially defined liquid media is a carry-over from BG MIL SPEC[7]. It will produce adequate numbers of spores (greater than 60%) in a relatively short period of time (Figure 2). The formulation as written in the MIL SPEC is antiquated and based on calculation of nitrogen level after digestion. Raw enzymatic digestion of animal products for media preparation is no longer regularly conducted by researchers, but rather, by component manufacturers. This complicated process will likely lead to inconsistencies from batch to batch. In lieu of this fact, we used pre-digested pancreatic digest of casein (BD-Difco, Cockeysville, MD). In reconstructing the media formulation, we calculated the amount of pancreatic digest of casein used on the nitrogen content as reported by the manufacturer's certificate of analysis. Of the three media recipes tested, processing the cell pellet produced by CD during the washing procedure was the most difficult. It was impossible to completely resuspend the pellet. This resulted in lower %VS/g numbers and lower overall yield, which was not entirely unexpected.

3.1.3 CDSM.

A chemically defined medium is preferable to a medium containing animal products due to the fact that ingredients do not tend to vary as much in content from lot to lot, which may explain inconsistencies seen in previous media studies.

Liquid media are easier to work with on a larger scale, as they are adaptable for use in fermentation vessels. 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 refractile bodies.[6] Vegetative cells show refractility upon initiation of sporulation, however, since fully formed and released spores are necessary, refractility by itself is not a sufficient measure for our requirements. Therefore, percent sporulation was determined by released spores seen using Schaeffer-Fulton spore stain method under 1000x light microscopy. Although CDSM induces endospores in a relatively short period of time when compared to the CD and NSM, the majority of endospored cells did not completely sporulate as per the criteria before lysis. Optimization and analysis of CDSM relative to seed culture and inoculum continues. Furthermore, investigations are

ongoing with variations of CDSM, with regard to components, methods and specific *Bacillus* strain, to elucidate modifications for achieving complete sporulation by our criteria and thus useful, aerosolizable spores for use as suitable simulants.

3.2 Growth Time in Number of Days and Percent Sporulation.

Tables 1-4 outline the percent sporulation of each strain tested the medium used and the number days growth.

Table 1. Percent sporulation and days growth from 29 April 2002

Media	BG	Days	% Spore
CD	10-88	9	70
CDSM 30	9372	0/10	0
CDSM 37	9372	10	0
CDSM 30	10-88	9	70
CDSM 37	10-88	0	0
NSM	9372	10	80
NSM	10-88	4	95

CDSM was grown at 30 and 37 degrees Celsius in an attempt to delineate a difference. No significant differences were observed, as three of the four cultures showed growth of cells and lysis prior to formation of spores.

Table 2. Percent sporulation and days growth from 29 May 2002

Media	BG	Days	% Spore
CD	9372	9	60
CD	10-88	9	80
CDSM	9372	28h/13	0
CDSM	10-88	28h/13	0
NSM	9372	12	90
NSM	10-88	6	95

ATCC CDSM cells grew for a period of 14 days yielding no spores, spore stain indicated most of the vegetative cells had lysed and the samples were terminated.

Table 3. Percent sporulation and days growth from 11 June 2002

Media	BG	Days	% Spore
CD	9372	7	60
CD	10-88	7	70
CDSM	9372	40h/12	0
CDSM	10-88	40h/5	80
NSM	9372	12	80
NSM	10-88	5	90

ATCC CDSM cells grew for a period of 14 days yielding no spores, spore stain indicated most of the vegetative cells had lysed. The viable spore count for this batch was low at 10^8 cfu/gm; the plate counts were consistently low.

Table 4. Percent sporulation and days growth from 3 July 2002

Media	BG	Time	% Spore
CD	9372	14	15
CD	10-88	7	80
CDSM	9372	28h/14	0
CDSM	10-88	28h/11	50
NSM	9372	10	80
NSM	10-88	7	90

ATCC CDSM grew for a period of 14 days yielding no spores, spore stain indicated most of the vegetative cells had lysed. The viable spore count for this batch was significantly lower than previous batches at 10^7 cfu/gm; plate counts were consistently low.

3.3 QA/QC Analysis.

3.3.1 Aerodynamic Particle Sizer (APS) Size Distribution.

The median diameters of spores grown on the three media were comparable at approximately one micron, when 80-90,000 particles were measured (Appendix A). The size criterion of 1-5 microns is based on current doctrine[1, 2, 7] and requires that particles remain effectively aerosolized in this range. Particles larger than five microns are not effectively suspended in the air for long periods of time and are not efficiently inhaled. Respirable 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. Results of APS size measurements for spores from April 2003 growth, as compared to the DPG prepared spores are presented in Appendix A. Table 5 indicates that the Dugway spores do not differ significantly in particle size, from those grown on NSM, CDSM and CD.

Table 5. Particle Size comparison (in µm) of *Bacillus subtilis* var. *niger* spores grown on NSM, CDSM and CD compared to Dugway Lot 10-88, evaluated on TSI 3310 APS.

	Total Particles (10^5)	Median Diameter (µm)
NSM	0.92	1.107
CDSM	0.81	1.073
CD	0.84	0.994
Dug BG	0.80	0.905

3.3.2 Fourier Transfer-Infrared Spectroscopy (FT-IR) Measurements.

Spectroscopic data was gathered with the Travel*IR* comparing the two strains of *bacillus* on each of the three media and to the Dugway lot 10-88 BG[3] original preparation. The measurement of the NSM and CD samples, of both strains, indicate a

strong resemblance to the 10-88 lot produced by DPG. The CDSM samples show a variability that may have been caused by considerably lower sporulation percentages. Most of the cells did not completely sporulate and were harvested as vegetative or lysed cell debris. Upon completion of the heat-shock step, any vegetative material would have been rendered non-viable. Spore viability is not essential for measurement with the Travel*IR*, that is, the chemical and atomic structures remain the same. It is plausible that the results seen with the CDSM samples are indicative of a mixture of spore, endospore, and dead vegetative material. Results of FT-IR measurements for all spores are presented in Appendix B.

3.3.3 Electron Microscopy (EM).

A visualization of spores under 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%, shows fully-formed, un-agglomerated spores. The spectral analysis indicates no residual interferants are present in the spore preparation. To better illustrate the particle size and lack of true-agglomeration of the spores, the samples were first affixed to conductive carbon tape (SPI supplies, West Chester, PA) on aluminum mounts and examined with no further sample preparation after aerosolization by the APS. This preparation allowed the spores to be freely dispersed and free of any potential electro-static interactions resultant from the lyophilization process. Results of electron microscopic scans for all spores are presented in Appendix C.

3.3.4 Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction (PCR) was conducted with the Ruggedized Advanced Pathogen Identification Device, R.A.P.I.D. PCR rapid light-cycler model AP 0097 (Figure 3). The R.A.P.I.D. and the primer, for *Bacillus subtilis* var. *niger*, were manufactured and prepared by Idaho Technology Inc. (Salt Lake City, Utah). Data shows that significant amplification occurred for the positive control after approximately 22 cycles. The in-house spore samples evaluated with the R.A.P.I.D. included the Dugway strain grown on each of the three tested media and the ATCC strain on NSM and CD. All samples tested achieved significant amplification before 30 cycles, indicating the speciation on the sample to be the same as that of the template.

4. CONCLUSIONS

Methods for simulant growth must be established and standardized within the Research and Development (R&D) community as a whole. 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 detection and identification with instruments being developed. The primary objective of this study was to identify and evaluate the most efficient method and protocol for the standardization of spore simulant organisms. Another goal was to

elucidate a more up to date media than that listed in the Military Specification (MIL SPEC), or to modify an existing media to incorporate the desired characteristics; short growth time accompanied by high sporulation percentage, ease of preparation and harvesting, chemically defined. The spores must be of high quality and purity so as not to distract from their intended use. The media and processes should leave no gross or minimal media components or by products that could cause detection interferences, or would cause spore agglomeration that would render the spores less aerosolizable.

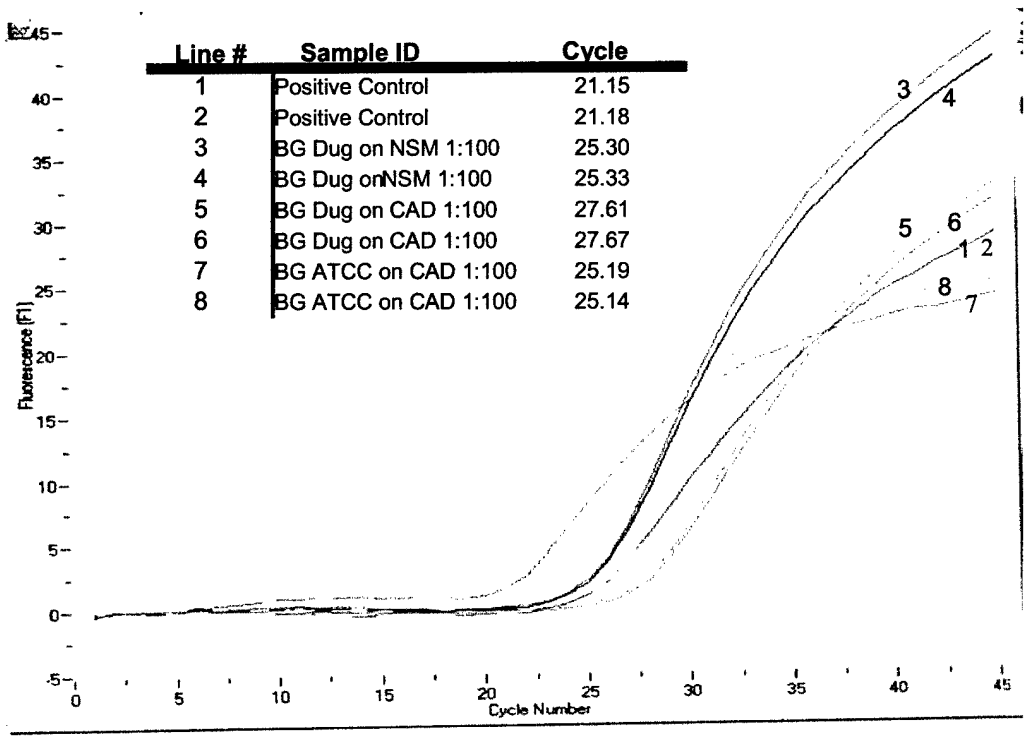

Figure 3. BG spore Identity with RAPID Light-Cycler (PCR)

Spores of the Dugway lot produced on Casein Digest (CD) and nutrient sporulation medium (NSM) using these methods are acceptable for use as a standardized simulant. Spores from the American Type Culture Collection (ATCC) strain were not effectively induced to meet the criteria on CD and chemically defined sporulation medium (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. CDSM is a very promising media as it is a liquid media and completely chemically defined. Continuing optimization of the CDSM seed inoculum is ongoing, along with refinement of the processing procedures. Table 6 outlines the complete growth trials utilizing each of the evaluated media; growth time, percent sporulation and viability, contamination and yield in milligrams.

Table 6. Comparison of Growth time, percent sporulation and viability, evidence of contamination, and total yield in milligrams between media formulations and *Bacillus* strain

Media	BG	Inoc Date	# Days growth	% Spore	% VS/gram	% ACC	# Vials	Yield (mg)
CD	9372	12802	10	85	Not tested	0	1	23.65
NSM	9372	12802	8	>90	Not tested	0	4	56.58
CD	9372	21902	14	10	Not tested	0	0	0
NSM	9372	21902	13	>90	Not tested	0	6	106.16
CD	9372	30502	10	85	Not tested	0	5	146.87
NSM	9372	30502	7	>90	No tested	0	6	143.47
CD	10-88	41602	3	85	1.2×10^{12}	0	6	523.8
CDSM	10-88	41602	12	10/80*en*	4.4×10^{11}	0	6	268.18
NSM	10-88	41602	3	90	9.3×10^{11}	0	7	943.81
CD	9372	42902	0	x	Discarded	n/a	0	0
CD	10-88	42902	9	70	1.4×10^{12}	0	7	548.1
CDSM 30°	9372	42902	10	0	No spores	0	0	0
CDSM 37°	9372	42902	10	0	No spores	0	0	0
CDSM 30°	10-88	42902	9	70	No spores	0	0	0
CDSM 37°	10-88	42902	14	0	No spores	0	0	0
NSM	9372	42902	10	80	9.7×10^{14}	0	5	981.7
NSM	10-88	42902	4	95	1.2×10^{12}	0	9	319.5
CD	9372	52902	9	60	$< 10 \times 10^{10}$	0	2	64.7
CD	10-88	52902	9	80	3.21×10^{12}	0	7	861.82
CDSM	9372	52902	14	0	$< 10 \times 10^{10}$	0	1	34.6
CDSM	10-88	52902	14	0	$< 10 \times 10^{9}$	0	1	69.25
NSM	9372	52902	12	90	5.53×10^{12}	0	5	498.97
NSM	10-88	52902	6	95	1.15×10^{14}	0	11	1057.71
CD	9372	61102	7	50	4.7×10^{11}	0	5	19.8
CD	10-88	61102	7	70	3.6×10^{12}	0	9	586.9
CDSM	9372	61102	14	0	No spores	0	3	52.33
CDSM	10-88	61102	5	80	1.2×10^{13}	0	6	106.13
NSM	9372	61102	12	80	7.3×10^{12}	0	7	200.78
NSM	10-88	61102	5	90	9.7×10^{12}	0	9	1062.12
CD	9372	70302	7	80	4.46×10^{10}	0	2	58.62
CD	10-88	70302	14	<25	1.2×10^{9}	0	9	757.92
CDSM	9372	70302	14	<10	4.99×10^{7}	0	4	196.68
CDSM	10-88	70302	12	<50	6.12×10^{12}	0	4	119.93
NSM	9372	70302	9	80	1.61×10^{12}	0	3	224.0
NSM	10-88	70302	6	90	4.47×10^{12}	0	3	114.92

en indicates endospores; incomplete sporulation
30° and 37° indicates temperature in degrees C, respectively

The establishment of the QA/QC guidelines, on a broader Joint-Services level, is also underway with the establishment of a Joint Services Simulant Working Group designed to address the needs of the joint community with regard to simulants, both Chemical and Biological. The Working Group was held as a series of meetings, from August 2002 through October 2002, with representatives from the DOD community tasked to design a recommended Plan of Action (POA) to the Joint Services Chemical Biological Technical Panel (JS CB Tech Panel) for insertion into the FY05-10 POM Budget. The working sub-groups addressed the following areas of concern; Simulant Needs, Simulant Selection and Simulant Development.

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GLOSSARY

APS	Aerodynamic Particle Sizer
ATCC	American Type Culture Collection
BG	*Bacillus atrophaeus,* formerly *Bacillus subtilis* var. *niger* , and formerly *Bacillus globigii*
BW	Biological Warfare
CD	Casein Digest
CDSM	Chemically Defined Sporulation Medium
dDI	Distilled Deionized Water
NSM	Nutrient Sporulation Medium
NBC	Nuclear, Biological and Chemical
PCR	Polymerase Chain Reaction
R.A.P.I.D.	Ruggedized Advanced Pathogen Identification Device
SEM	Scanning Electron Microscopy
TARA	Threat Agent Reduction Agency
%VS per g	Percent Viable Spores, per gram
%ACC	Percent Atypical Colony Count

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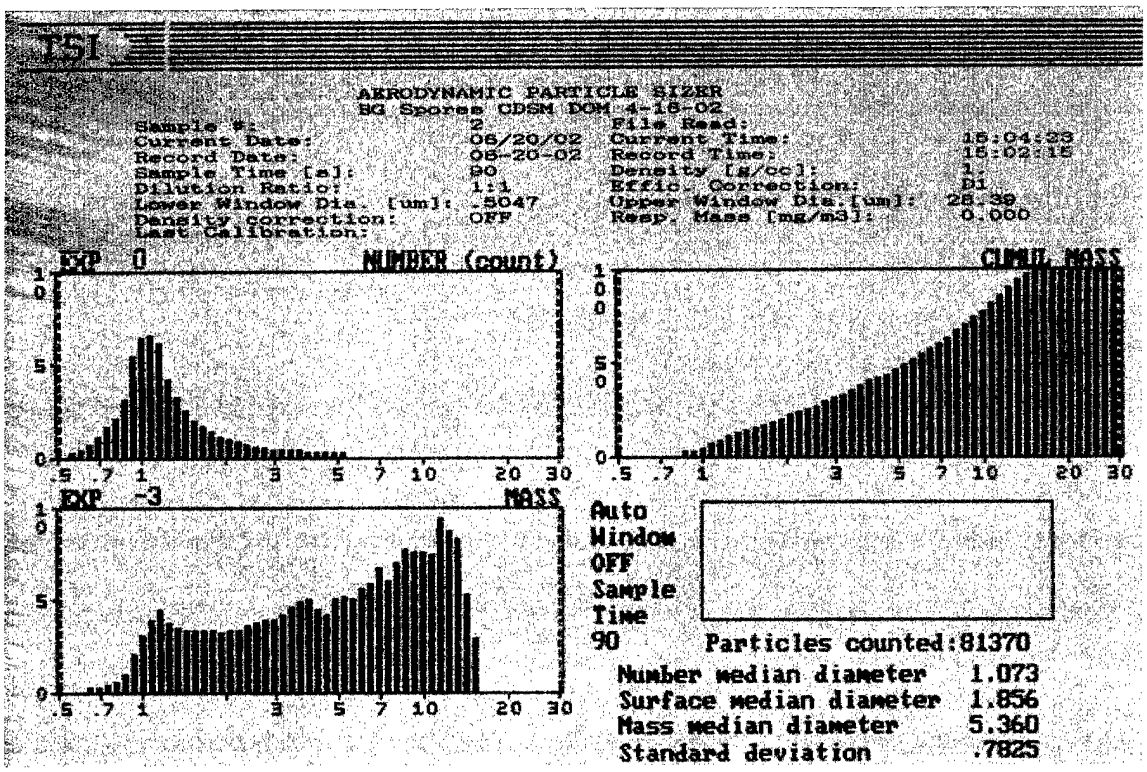
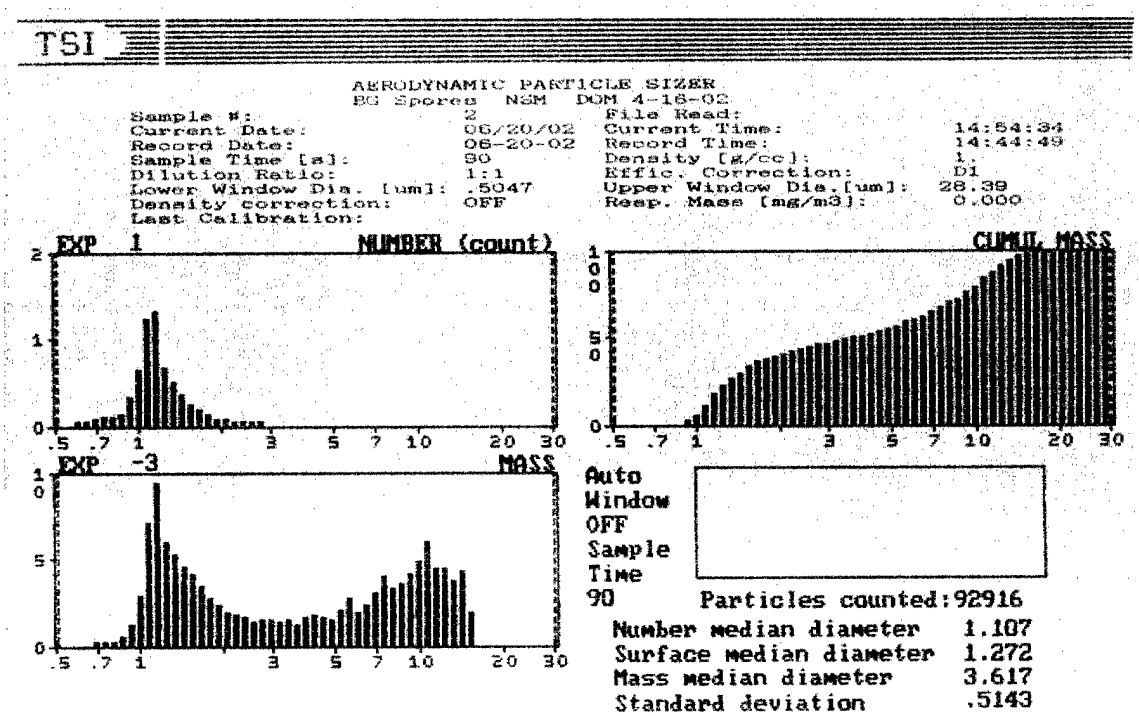
APPENDIX A

AERODYNAMIC PARTICLE SIZING TEST RESULTS
COMPARISON OF SPORES GROWN IN CDSM, NSM AND CD TO DPG
PRODUCED LOT 10-88.

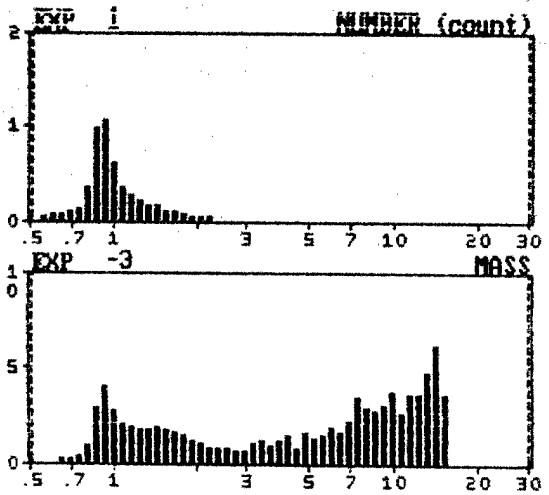


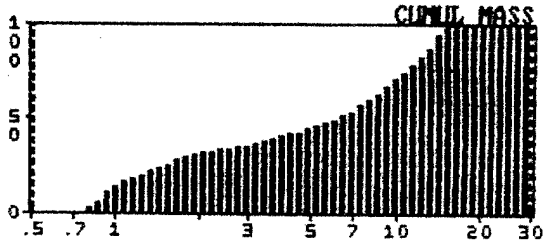


TSI

AERODYNAMIC PARTICLE SIZER
BG Spores Dugway

Sample #:	2	File Read:	
Current Date:	06/13/02	Current Time:	16:29:11
Record Date:	06-13-02	Record Time:	16:36:58
Sample Time [s]:	45	Density [g/cc]:	1.
Dilution Ratio:	1:1	Effic. Correction:	D1
Lower Window Dia. [um]:	.5047	Upper Window Dia.[um]:	28.39
Density correction:	OFF	Resp. Mass [mg/m3]:	0.000
Last Calibration:			





Auto Window OFF
Sample Time 45

Particles counted: 36262

Number median diameter	.9108
Surface median diameter	1.166
Mass median diameter	6.120
Standard deviation	.5302

TSI

AERODYNAMIC PARTICLE SIZER
BG Spores CAD DOM 4-16-02

Sample #:	2	File Read:	
Current Date:	06/20/02	Current Time:	15:21:10
Record Date:	06-20-02	Record Time:	15:14:48
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Dilution Ratio:	1:1	Effic. Correction:	D1
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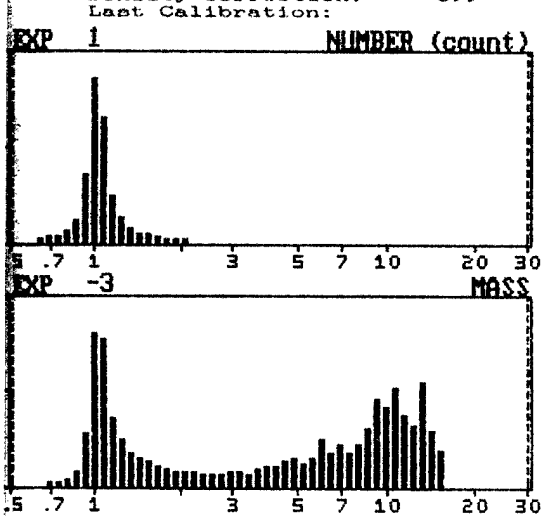
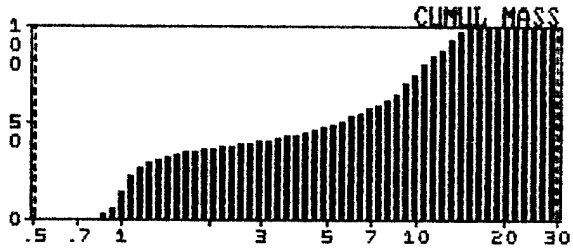
Density correction:	OFF	Resp. Mass [mg/m3]:	0.000
Last Calibration:			





Auto Window OFF
Sample Time 90

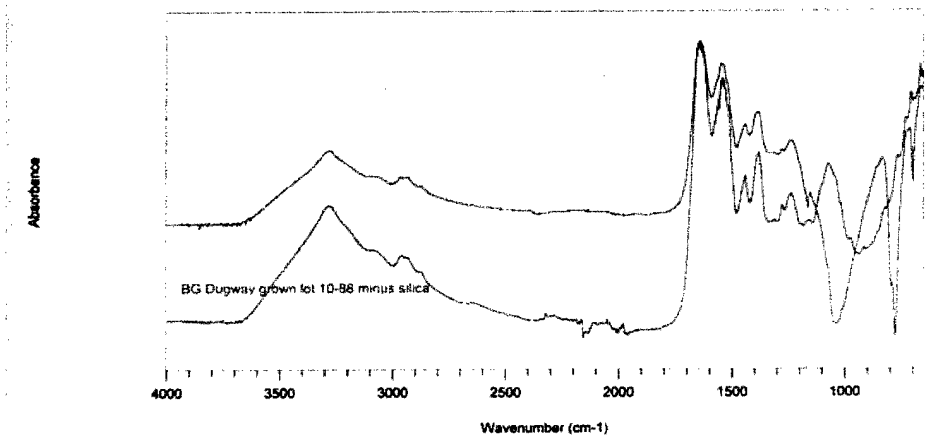
Particles counted: 84350

Number median diameter	.9940
Surface median diameter	1.066
Mass median diameter	5.396
Standard deviation	.4621

APPENDIX B

FT-IR RESULTS COMPARING SPORES GROWN ON NSM, CD AND CDSM TO DPG PRODUCED LOT 10-88.

specid.spc Dugway 10-88 BG grown on NSM, AMSSB-RRT-BT, DOM 061102

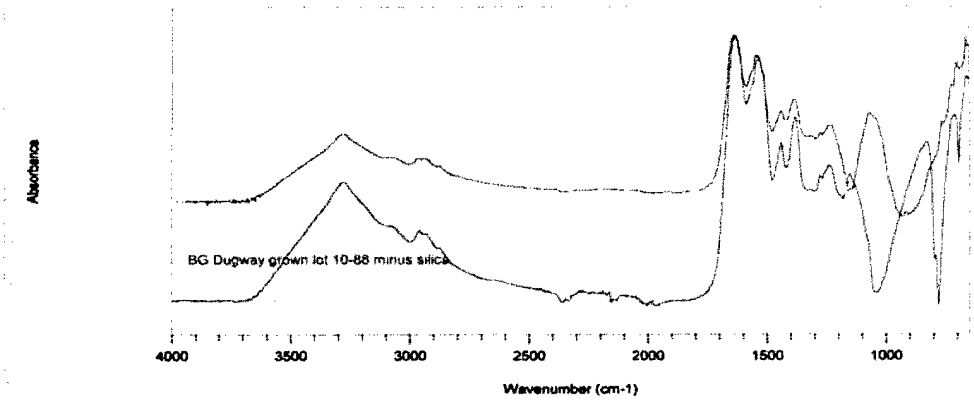


BG Dugway on NSM compared to BG 10-88 minus silica

Hit #	Quality	Library	Memo
1	0.000029	test.lib	Dugway 10-88 BG grown on NSM, AMSSB-RRT-BT, DOM 0
2	0.009546	test.lib	Dugway 10-88 BG grown on CAD, AMSSB-RRT-BT, DOM 0
3	0.168442	test.lib	ATCC 9372 grown on CAD, AMSSB-RRT-BT, DOM 061102
4	0.179062	test.lib	Dugway BG lot 10-88 grown on CDSM, AMSSB-RRT-BT, DC
5	0.250785	test.lib	BG Dugway lot 10-88, neat
6	0.576679	test.lib	ATCC 9372 grown on CDSM, AMSSB-RRT-BT, DOM 06110
7	0.601328	test.lib	isopropanol solution test
8	0.818135	test.lib	BG Dugway grown lot 10-88 minus silica
9	0.847855	test.lib	silica
10	0.891005	test.lib	200 Mesh Silica, neat

1. DPG strain grown on NSM (top trace) compared to DPG lot 10-88 BG (bottom trace) as grown and prepared by DPG.

specid.spc ATCC 9372 BG spores grown on NSM, DOM 061102 (vial red 34) 3.58 mg

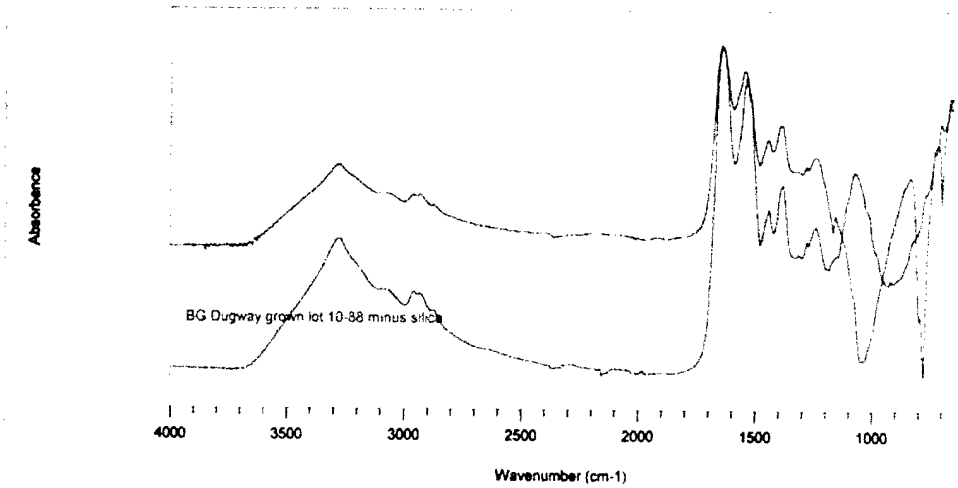


BG ATCC 9372 on NSM compared to BG lot 10-88 minus silica

Hit #	Quality	Library	Memo
1	0.007009	test.lib	Dugway 10-88 BG grown on CAD, AMSSB-RRT-BT, DOM 0
2	0.014127	test.lib	Dugway 10-88 BG grown on NSM, AMSSB-RRT-BT, DOM 0
3	0.112736	test.lib	ATCC 9372 grown on CAD, AMSSB-RRT-BT, DOM 061102
4	0.124144	test.lib	Dugway BG lot 10-88 grown on CDSM, AMSSB-RRT-BT, DC
5	0.182791	test.lib	BG Dugway lot 10-88, neat
6	0.494179	test.lib	ATCC 9372 grown on CDSM, AMSSB-RRT-BT, DOM 06110
7	0.610579	test.lib	isopropanol solution test
8	0.695462	test.lib	BG Dugway grown lot 10-88 minus silica
9	0.785225	test.lib	silica
10	0.830776	test.lib	200 Mesh Silica, neat

2. ATCC 9372 BG grown on NSM (top trace) compared to DPG lot 10-88 BG (bottom trace) as grown and prepared by DPG.

specid.spc Dugway 10-88 BG grown on CAD, AMSSB-RRT-BT, DOM 061102

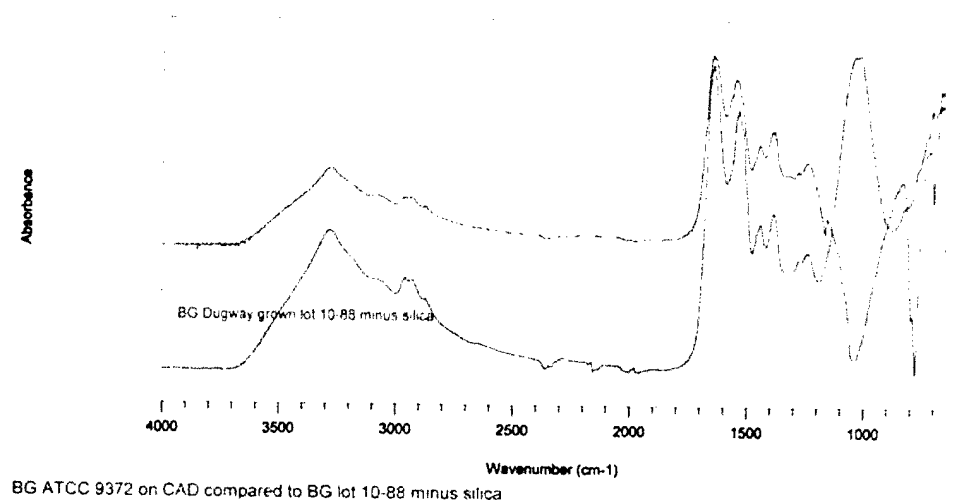


BG Dugway on CAD compared to BG 10-88 minus silica

Hit #	Quality	Library	Memo
1	0.000023	test.lib	Dugway 10-88 BG grown on CAD, AMSSB-RRT-BT DOM 0
2	0.009596	test.lib	Dugway 10-88 BG grown on NSM, AMSSB-RRT-BT, DOM 0
3	0.123342	test.lib	ATCC 9372 grown on CAD, AMSSB-RRT-BT, DOM 061102
4	0.154465	test.lib	Dugway BG lot 10-88 grown on CDSM, AMSSB-RRT-BT, D
5	0.201219	test.lib	BG Dugway lot 10-88, neat
6	0.52246	test.lib	ATCC 9372 grown on CDSM, AMSSB-RRT-BT, DOM 06110
7	0.642714	test.lib	isopropanol solution test
8	0.661988	test.lib	BG Dugway grown lot 10-88 minus silica
9	0.811033	test.lib	silica
10	0.854878	test.lib	200 Mesh Silica, neat

3. DPG strain grown on CD (top trace) compared to DPG lot 10-88 BG (bottom trace) as grown and prepared by DPG.

specid.spc ATCC 9372 grown on CAD, AMSSB-RRT-BT, DOM 061102

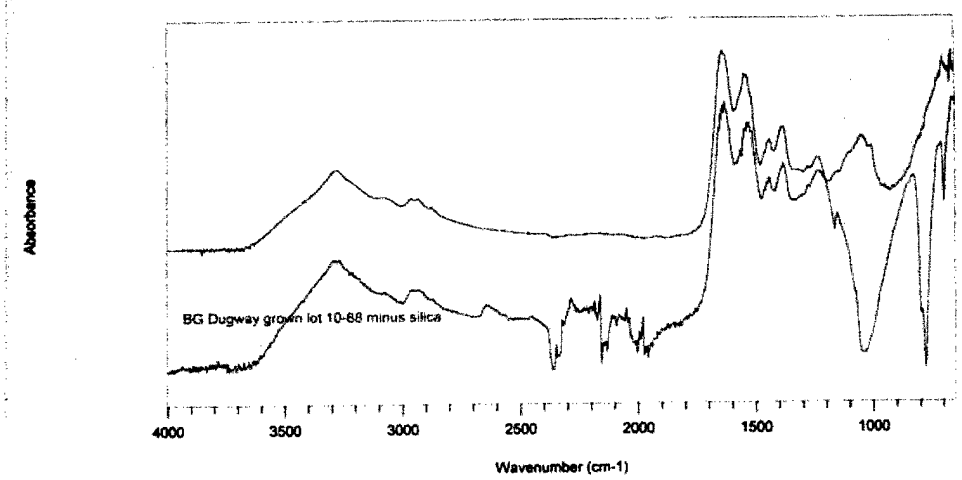


BG ATCC 9372 on CAD compared to BG lot 10-88 minus silica

Hit #	Quality	Library	Memo
1	0.000018	test.lib	ATCC 9372 grown on CAD, AMSSB-RRT-BT, DOM 061102
2	0.095501	test.lib	BG Dugway lot 10-88, neat
3	0.12318	test.lib	Dugway 10-88 BG grown on CAD, AMSSB-RRT-BT, DOM 0
4	0.15764	test.lib	Dugway BG lot 10-88 grown on CDSM, AMSSB-RRT-BT, D
5	0.168461	test.lib	Dugway 10-88 BG grown on NSM, AMSSB-RRT-BT, DOM 0
6	0.193402	test.lib	ATCC 9372 grown on CDSM, AMSSB-RRT-BT, DOM 06110
7	0.61497	test.lib	200 Mesh Silica, neat
8	0.643839	test.lib	silica
9	0.654694	test.lib	isopropanol solution test
10	0.901532	test.lib	BG Dugway grown lot 10-88 minus silica

4. ATCC 9372 BG grown on CD (top trace) compared to DPG lot 10-88 BG (bottom trace) as grown and prepared by DPG.

specid.spc Dugway BG lot 10-88 grown on CDSM, AMSSB-RRT-BT, DOM 061102

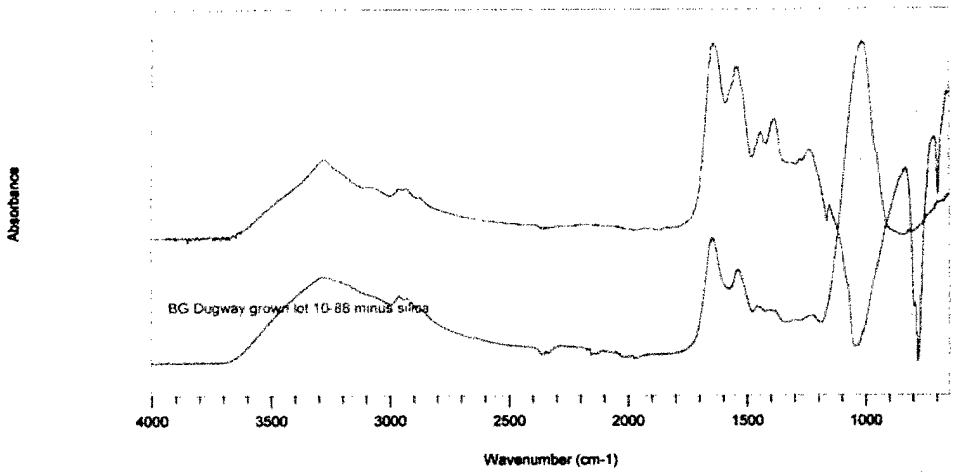


BG Dugway on CDSM compared to BG 10-88 minus silica

Hit #	Quality	Library	Memo
1	0.000074	test.lib	Dugway BG lot 10-88 grown on CDSM, AMSSB-RRT-BT, DC
2	0.153825	test.lib	Dugway 10-88 BG grown on CAD, AMSSB-RRT-BT, DOM 0
3	0.157254	test.lib	ATCC 9372 grown on CAD, AMSSB-RRT-BT, DOM 061102
4	0.178418	test.lib	Dugway 10-88 BG grown on NSM, AMSSB-RRT-BT, DOM 0
5	0.197378	test.lib	BG Dugway lot 10-88, neat
6	0.43309	test.lib	ATCC 9372 grown on CDSM, AMSSB-RRT-BT, DOM 06110
7	0.657764	test.lib	silica
8	0.683165	test.lib	isopropanol solution test
9	0.730157	test.lib	200 Mesh Silica, neat
10	0.840659	test.lib	BG Dugway grown lot 10-88 minus silica

5. DPG strain grown on CDSM (top trace) compared to DPG lot 10-88 BG (bottom trace) as grown and prepared by DPG.

specid.spc ATCC 9372 grown on CDSM, AMSSB-RRT-BT, DOM 061102



BG ATCC 9372 on CDSM compared to BG lot 10-88 minus silica

Hit #	Quality	Library	Memo
1	0.000037	test.lib	ATCC 9372 grown on CDSM, AMSSB-RRT-BT, DOM 06110
2	0.193156	test.lib	ATCC 9372 grown on CAD, AMSSB-RRT-BT, DOM 061102
3	0.263724	test.lib	BG Dugway lot 10-88, neat
4	0.333439	test.lib	200 Mesh Silica, neat
5	0.433246	test.lib	Dugway BG lot 10-88 grown on CDSM, AMSSB-RRT-BT, DC
6	0.494232	test.lib	silica
7	0.522295	test.lib	Dugway 10-88 BG grown on CAD, AMSSB-RRT-BT, DOM 0
8	0.576747	test.lib	Dugway 10-88 BG grown on NSM, AMSSB-RRT-BT, DOM 0
9	0.736499	test.lib	isopropanol solution test
10	0.993438	test.lib	BG Dugway grown lot 10-88 minus silica

6. ATCC 9372 BG grown on CDSM (top trace) compared to DPG lot 10-88 BG (bottom trace) as grown and prepared by DPG.

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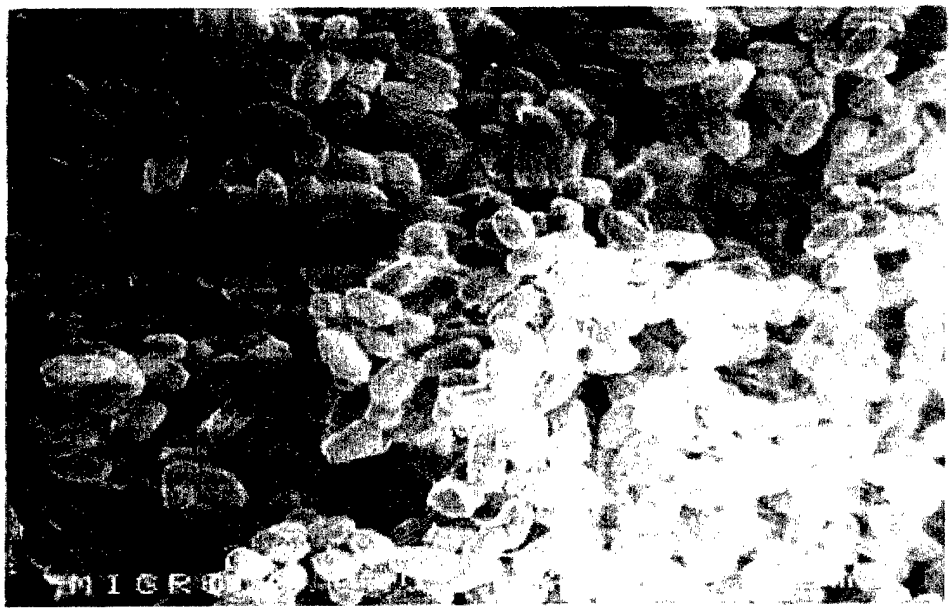
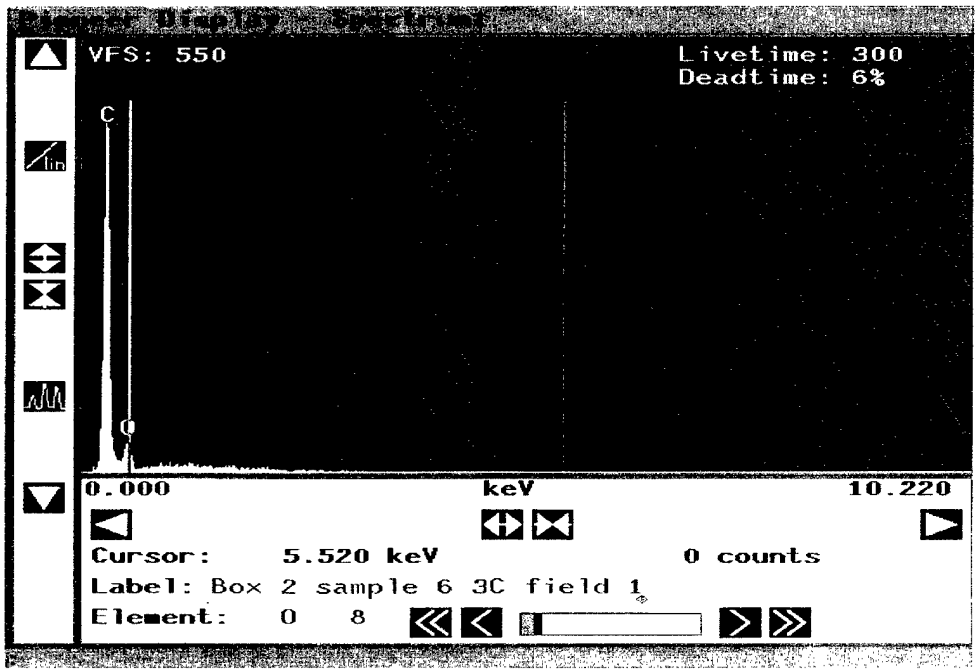
APPENDIX C

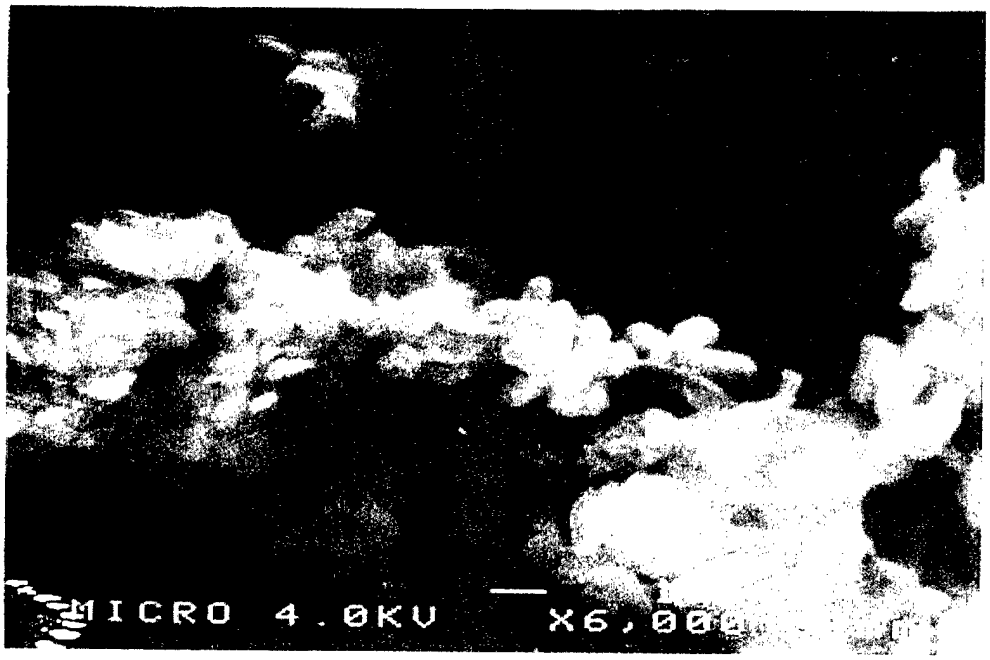
ELECTRON MICROGRAPHS AND ELEMENTAL ANALYSIS
OF SPORES GROWN ON NSM, CD AND CDSM.



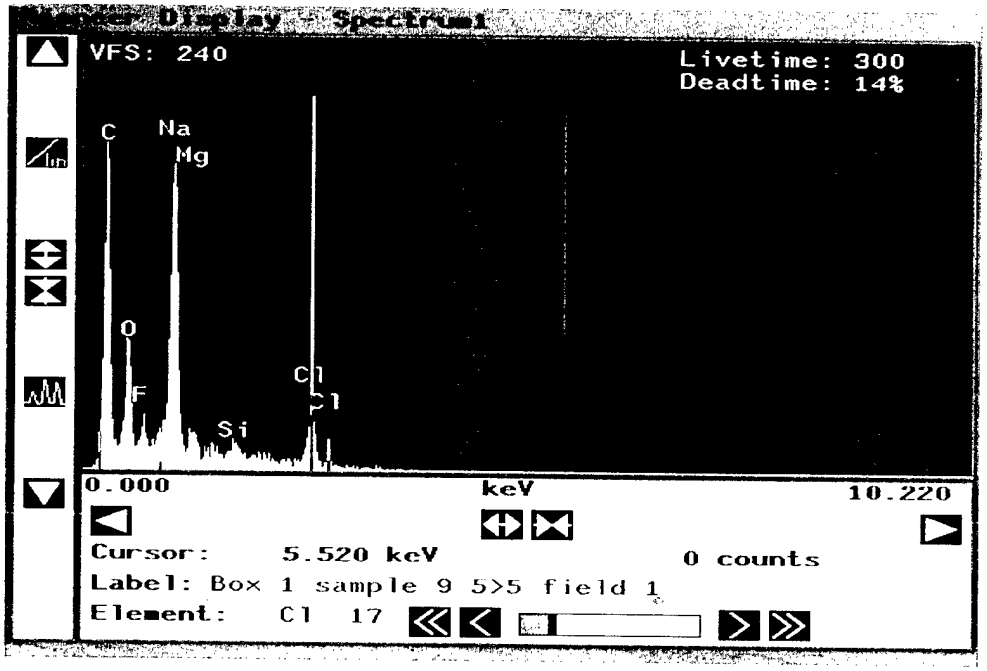
DPG Bioferm Lot 10-88 (BG) on NSM; SEM shows spores of approximately 1 µm.



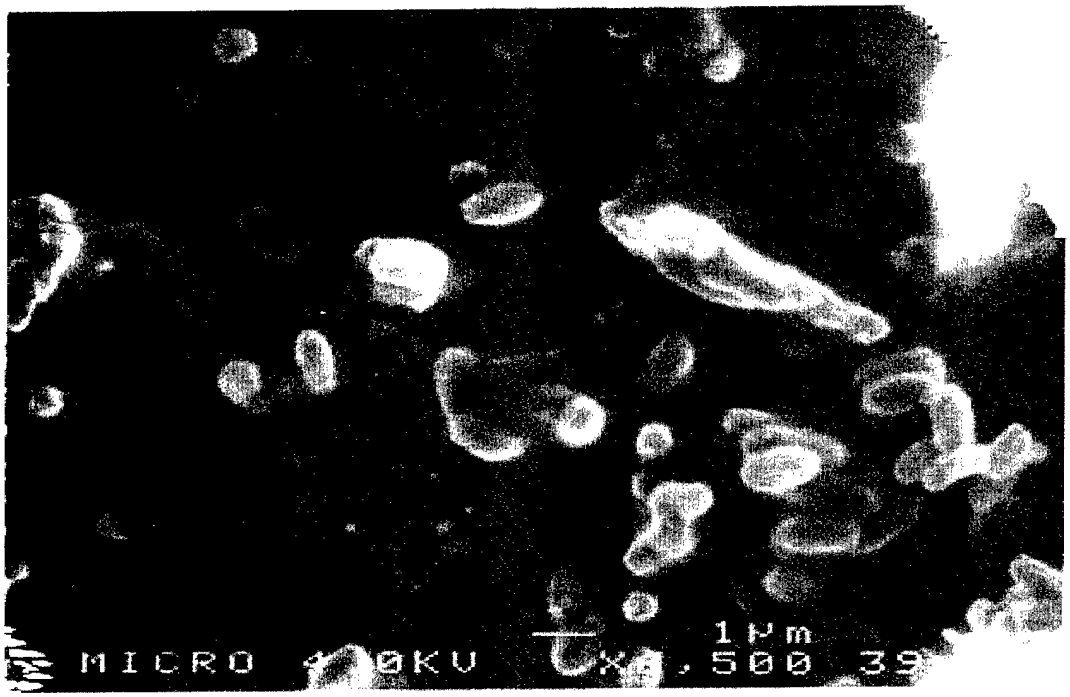
Elemental Analysis of DPG Bioferm Lot 10-88 (BG) grown on NSM shows Carbon (C) as the primary component.



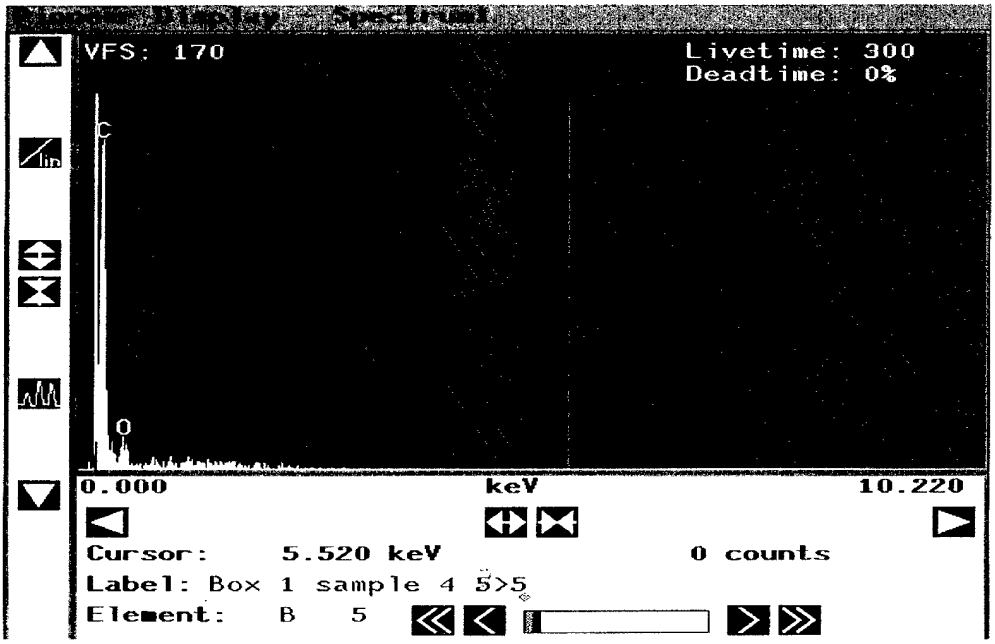
DPG Bioferm Lot 10-88 (BG) in CD; SEM shows spores of approximately 1 µm. There are less total spores in the preparation as compared to the NSM grown spores.



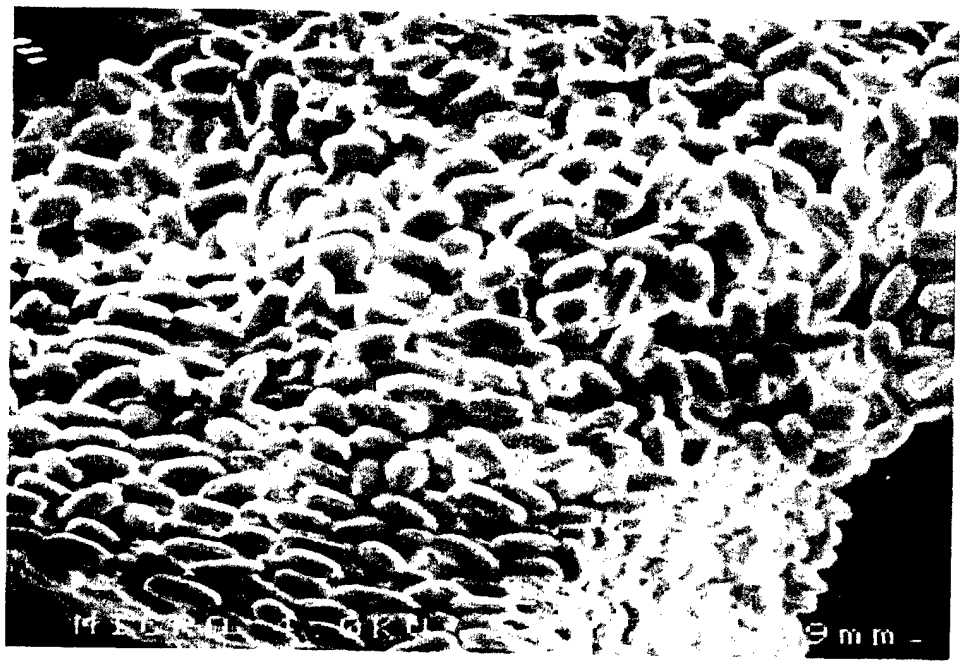
Elemental Analysis of DPG Bioferm Lot 10-88 (BG) grown in CD indicates other elements present in the preparation; of interest are sodium (Na), magnesium (Mg), and chlorine (Cl) as these are components of the CD media.



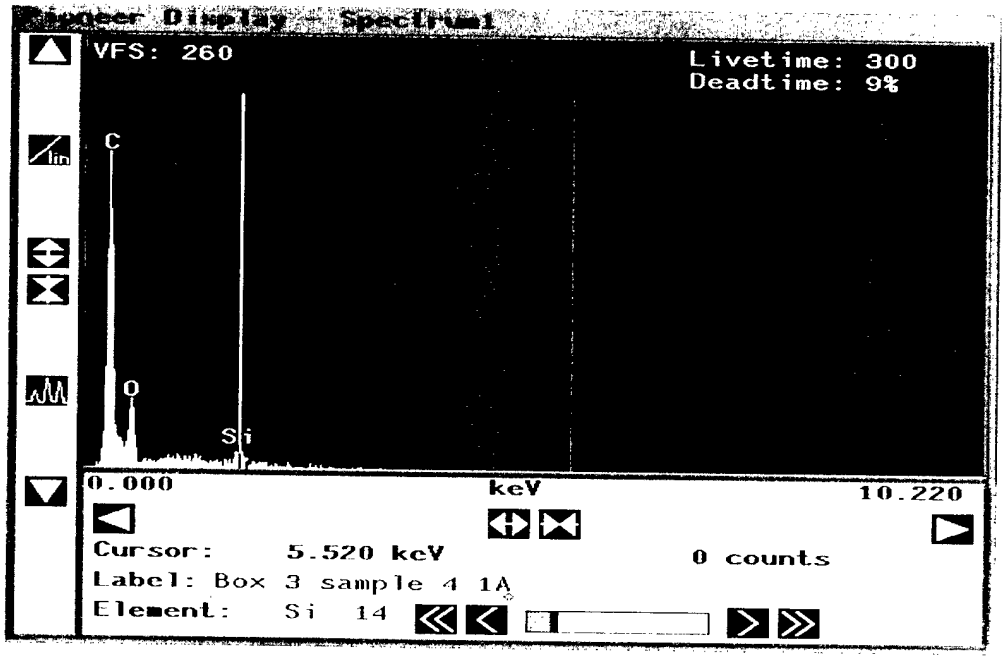
DPG Bioferm Lot 10-88 (BG) in CDSM; SEM shows spores of approximately 1 µm.



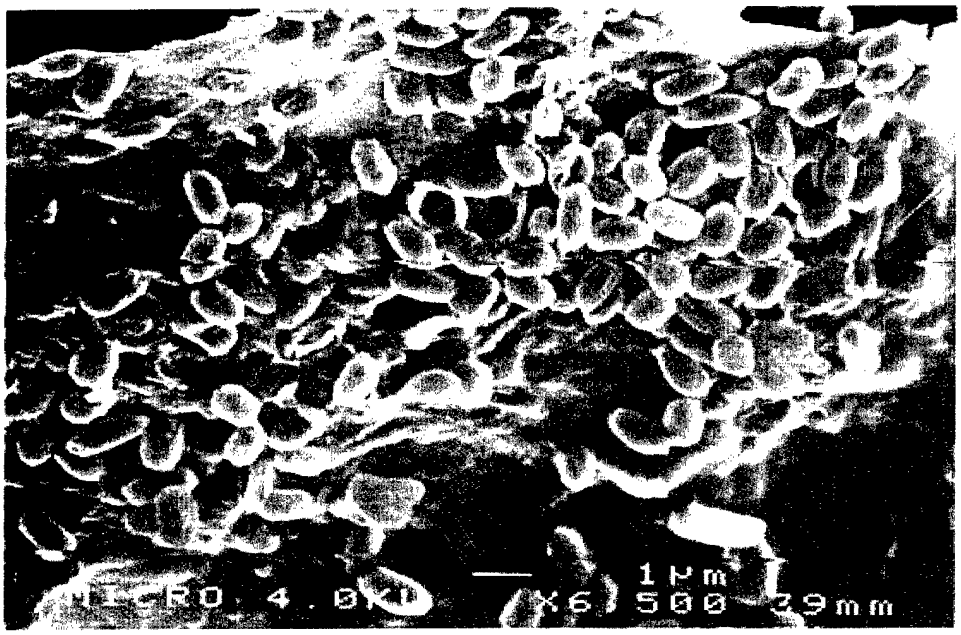
Elemental Analysis of DPG Bioferm Lot 10-88 (BG) grown in CDSM indicates Carbon (C) as the primary component.



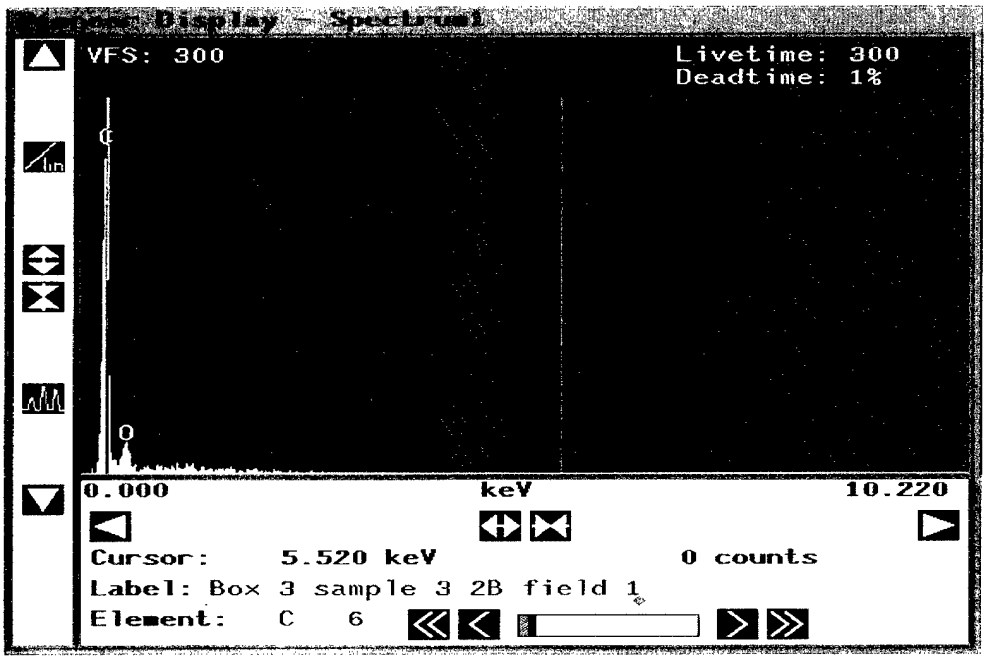
Bacillus subtilis var. *niger*, ATCC 9372 on NSM; SEM shows spores of approximately 1 µm.



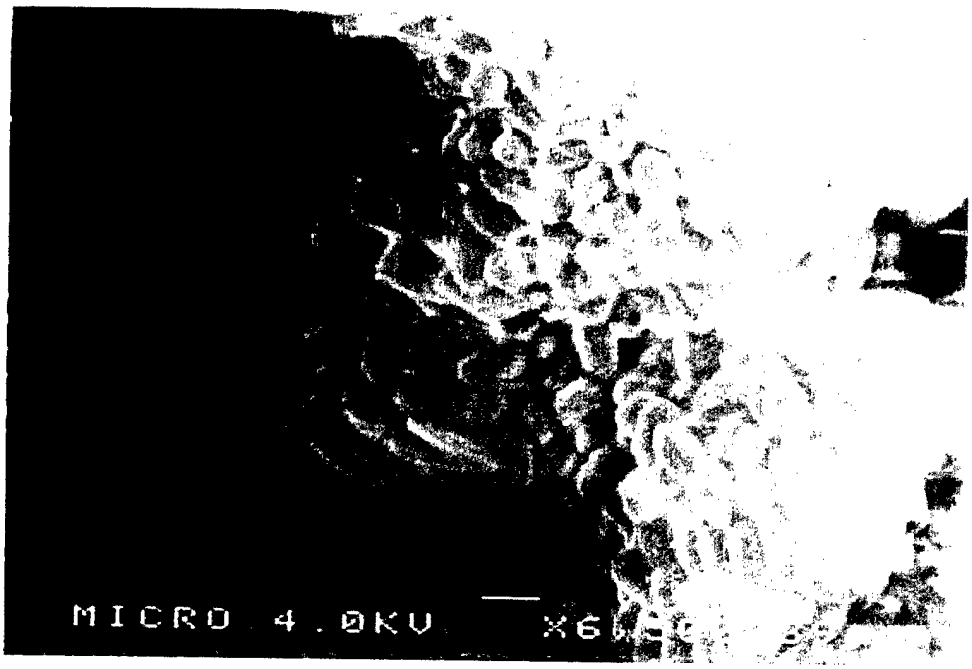
Elemental Analysis of *Bacillus subtilis* var. *niger*, ATCC 9372 on NSM indicates Carbon (C) as the primary component, with trace amount of silicon (Si) (additional line over Si peak was computer generated after analysis to indicate location/presence of compound).



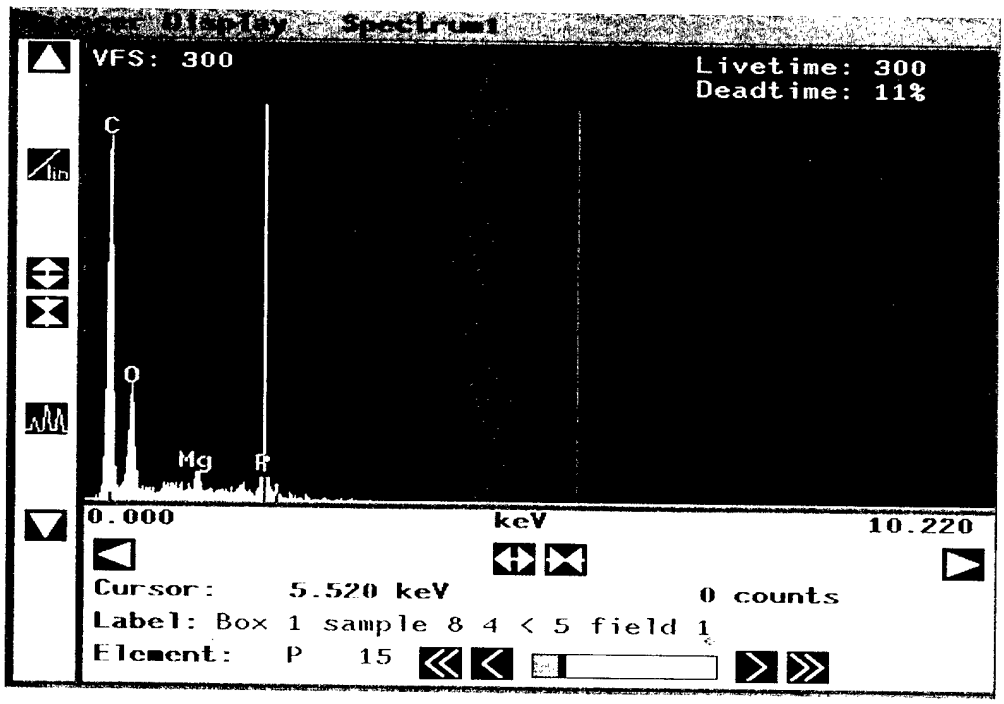
Bacillus subtilis var. *niger*, ATCC 9372 in CD; SEM shows spores of approximately 1 µm.



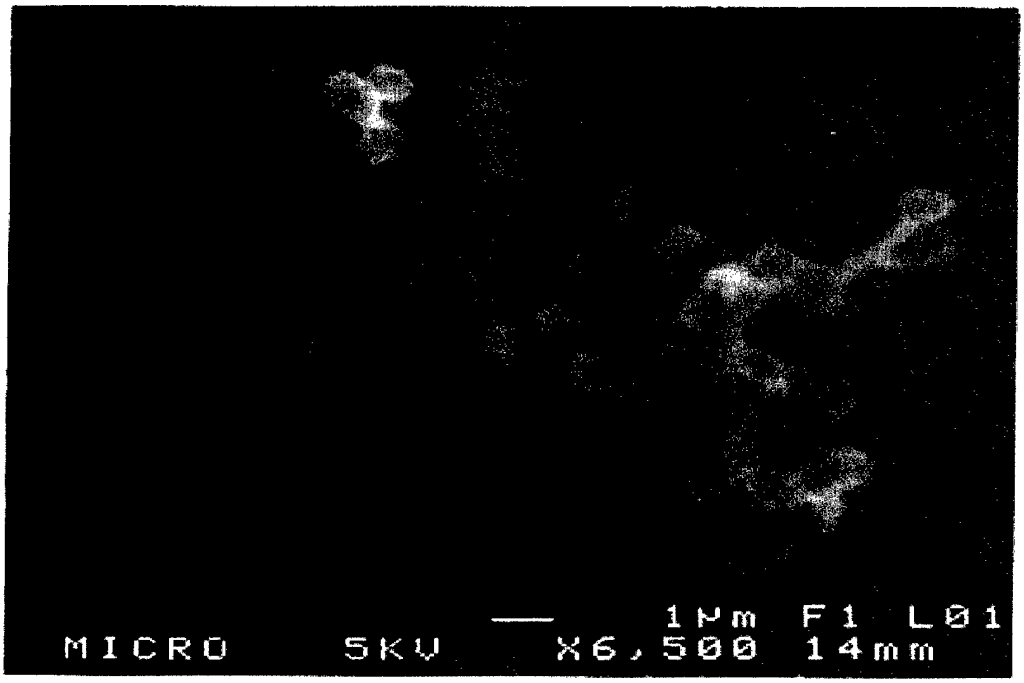
Elemental Analysis of *Bacillus subtilis* var. *niger*, ATCC 9372 in CD indicates Carbon (C) as the primary component, other trace elements are not present in this strain preparation of CD (see page C-8).

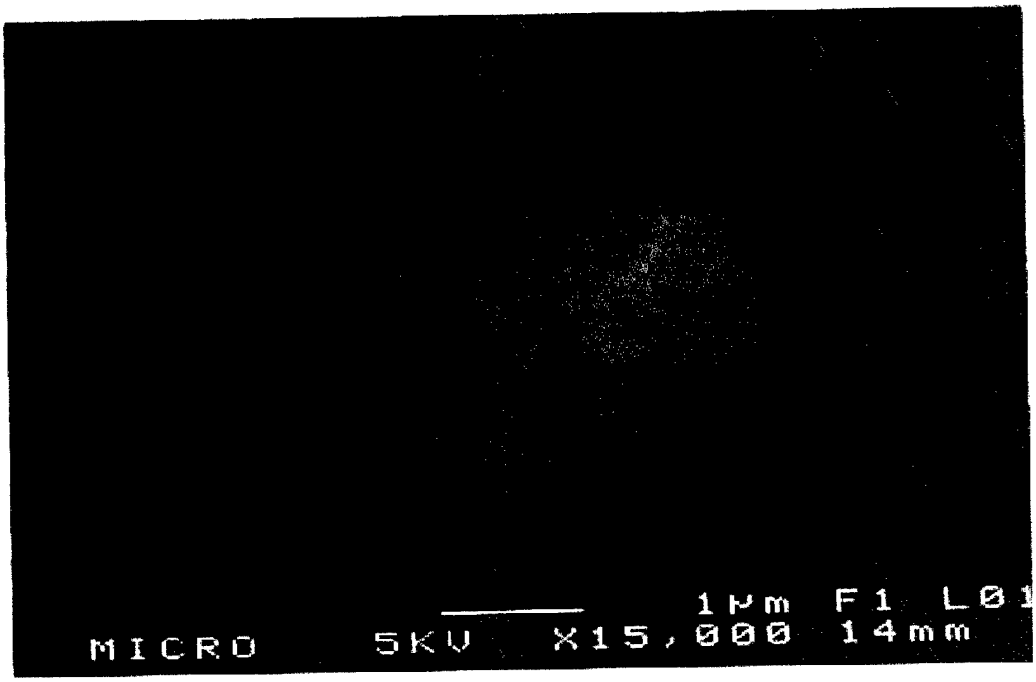


Bacillus subtilis var. *niger*, ATCC 9372 in CDSM; SEM shows spores of approximately 1 µm.

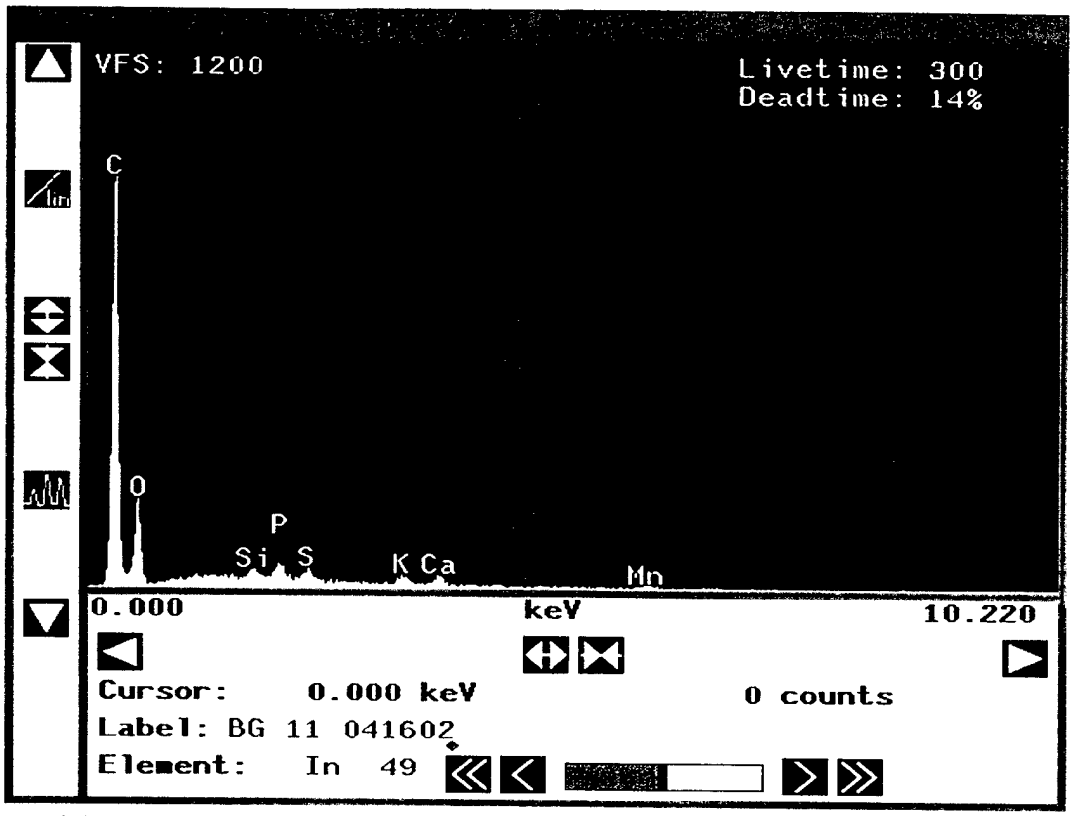


Elemental Analysis of *Bacillus subtilis* var. *niger*, ATCC 9372 in CDSM indicates Carbon (C) as the primary component, trace amounts of magnesium (Mg) and phosphorus (P) (additional line over Si peak was computer generated after analysis to indicate location/presence of compound).





DPG Bioferm Lot 10-88 (BG) in CDSM; SEM shows spores of approximately 1 µm. Spore preparations were not aerosolized with the APS prior to scans.



Elemental Analysis of DPG Bioferm Lot 10-88 (BG) grown in CD indicates additional elements present in the preparation; of interest are; manganese (Mn), potassium, (K), sulphur (S), calcium (Ca), and phosphorous (P), these are components of the CD media.