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Standard Practice for Characterization of *Bacillus* Spore Suspensions for Reference Materials[1]

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INTRODUCTION

Bacillus spp. are aerobic, rod-shaped, Gram positive bacteria that produce endospores under nutrient limiting conditions. The endospores are designed to persist in extreme environments and consequently are highly resistant to inactivation by heat, chemicals and irradiation. A few species of *Bacillus* are medically important because of their impact on human and animal health while others have important agricultural and industrial applications. Measurement of viable *Bacillus* spores present in a suspension can be performed using classical microbiology techniques, such as growth on nutrient medium. The spore suspension is diluted, an aliquot spread on solid nutrient medium, incubated at an appropriate temperature, and the resulting colonies counted. The selection of the type of growth medium and incubation temperature for the optimal growth of a particular *Bacillus* species should be determined by consultation of relevant literature or by comparison of different growth media and incubation temperatures.

Bacillus spore reference materials have many important applications in agriculture, basic research, medical diagnosis, detector validation, and sterility testing. Uniform methods for the characterization of spores will improve the comparison of different lots of materials and results between different laboratories.

1. Scope

1.1 This practice is focused on two basic measurements to characterize *Bacillus* reference materials, the enumeration of spores using growth of colonies on nutrient media and using phase contrast microscopy to determine spore quality and homogeneity. Additional information on advanced methods for characterization is provided in Appendix X1.

1.2 This document will provide the user with recommendations for measurement methods, and the details and conditions that should be employed to ensure reliable and high-quality data are obtained. The practice will help ensure that results obtained from the characterization are reported in a uniform manner. This will allow others to replicate the measurements and facilitate the comparison of different lots of *Bacillus* spore suspensions used as reference materials. It is important to note that the *Bacillus* species are a heterogeneous group and their specific requirements for growth and sporulation may vary. Users of this practice are encouraged to consult the literature for specific information on the species of *Bacillus* bacteria they are using (**1**).[2]

1.3 This standard practice does not provide guidance for the identification of unknown species of bacteria. The identification of *Bacillus* species has been traditionally based on colony morphology, growth on selective media, and biochemical tests, but more recently nucleic acid technologies have enabled the phylogenetic analysis of this group based on 16S DNA sequence similarities (**1**).

1.4 Some *Bacillus* spp. are pathogenic to humans and animals and the user is advised to adhere to safe laboratory procedures and practices for handling spores from these species (**2**). This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use (**2**).

1.5 This practice assumes a basic knowledge of microbiology and molecular biology and access to the cited references.

[1] This practice is under the jurisdiction of ASTM Committee E54 on Homeland Security Applications and is the direct responsibility of Subcommittee E54.01 on CBRNE Detection and CBRN Protection.

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[2] The boldface numbers in parentheses refer to a list of references at the end of this standard.

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D1129 Terminology Relating to Water

D4455 Test Method for Enumeration of Aquatic Bacteria by Epifluorescence Microscopy Counting Procedure (Withdrawn 2019)[4]

D6974 Practice for Enumeration of Viable Bacteria and Fungi in Liquid Fuels—Filtration and Culture Procedures

E1873 Guide for Detection of Nucleic Acid Sequences by the Polymerase Chain Reaction Technique (Withdrawn 2014)[4]

E2197 Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Chemicals

E2414 Test Method for Quantitative Sporicidal Three-Step Method (TSM) to Determine Sporicidal Efficacy of Liquids, Liquid Sprays, and Vapor or Gases on Contaminated Carrier Surface (Withdrawn 2014)[4]

E2458 Practices for Bulk Sample Collection and Swab Sample Collection of Visible Powders Suspected of Being Biological Agents and Toxins from Nonporous Surfaces

2.2 *Standard Methods for the Examination of Water and Wastewater:*[5]

Method 9218 Aerobic Endospores (2007)

Method 9215 Heterotrophic Plate Count (2004)

Environmental Protection Agency Standard Procedure for Enumeration of Bacterial Inocula on Carriers (Carrier Counts) for the Germicidal Spray Products as Disinfectants Test, Disinfectant Towelette Test, and the Tuberculocidal Activity of Disinfectants Test SOP Number: MD-04-05 Date Revised: 01-13-09[6]

2.3 *ISO Standards:*[7]

ISO 4833:2003 Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of microorganisms – Colony-count technique at 30 degrees C

ISO 21528-1:2004 Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of Enterobacteriaceae – Part 1: Detection and enumeration by MPN technique with pre-enrichment

2.4 *United States Pharmacopeia Standards:*

USP. 2006 Microbiological Best Laboratory Practices. USP 29 Suppl 2 pp. 3804-3807

USP. 2003 Good Microbiological Laboratory Practices. Pharmacopeial Forum 29(3):842-850.

USP. 2004 Microbiological Best Laboratory Practices. Pharmacopeial Forum 29(3):1713-1721

3. Terminology

3.1 *Definitions:*

3.1.1 *colony forming unit (CFU), n*—units for the number of viable particles present in a solution. A CFU can result from a single viable bacterial cell or from a clump of cells. **(D1129)**

3.1.2 *vortex mixing, v*—applying a tube containing a liquid sample to a special laboratory mixer that establishes a vigorous circular motion in the bottom of the tube.

3.1.2.1 *Discussion*—The circular mixing motion results in a vortex in the tube that ensures the complete suspension of the entire tube contents.

4. Summary of Practice

4.1 *Viable Spore Concentration by Plating on Nutrient Agar*—Plating bacteria on nutrient media is a well-established method for detection of bacteria in water (**3**). Suspensions of spores are first mixed well by vortex mixing or pipetting up and down vigorously to insure homogeneity of the spore suspension and then serially diluted using an appropriate buffer. Three serial dilutions are prepared from a spore reference sample. An aliquot of the diluted spore suspension is placed on a nutrient agar plate and spread using aseptic techniques. After incubation, the colonies on the plates are counted and the numbers of viable spores are referred to as colony forming units (CFU). The average number of colonies obtained from the diluted suspension are used to calculate the concentration of the original stock solution and reported as CFU/mL. In order to obtain consistent results, careful attention must be paid to detail to ensure adequate dispersion of spores and avoiding losses during the process.

4.2 *Spore Quality and Homogeneity Determined by Phase Contrast Microscopy*—An inexpensive, rapid and effective method to determine quality and homogeneity of spore preparations. A drop of the spore sample is placed on a microscope slide and covered with a coverslip. The spore preparation is examined using a high power objective (typically 100×) with a phase contrast microscope. Phase bright spores, phase dark spores, phase dark vegetative cells, and spore clumps are counted either manually, using automated counting devices or by digital imaging and computer software techniques (**4**). The percentage of phase bright spores in the sample is calculated and reported.

5. Significance and Use

5.1 Standard practices for the characterization of spores used as reference materials are important to ensure a uniform

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[4] The last approved version of this historical standard is referenced on www.astm.org.

[5] Available from American Public Health Association, Standard Methods for the Examination of Water and Wastewater, Washington, DC 20001, http://www.standardmethods.org/.

[6] Available from United States Environmental Protection Agency (EPA), Ariel Rios Bldg., 1200 Pennsylvania Ave., NW, Washington, DC 20460, http://www.epa.gov.

[7] Available from International Organization for Standardization (ISO), 1, ch. de la Voie-Creuse, Case postale 56, CH-1211, Geneva 20, Switzerland, http://www.iso.ch.

basis for testing the performance of detection devices and laboratory instruments. *Bacillus* spore suspensions can be used for a large variety of purposes including testing environmental sampling techniques, inactivation methods, decontamination methods and basic research.

5.2 The practice is intended for both manufacturers and end users of *Bacillus* spore suspensions. The results of the characterization measurements are presented in a report of analysis (ROA). The ROA should provide sufficient detail about the measurement technique to enable the customers to replicate the measurements, allowing them to determine if the properties of the spore suspension changed during shipping and storage.

5.3 The enumeration of the viable spores and determination of homogeneity by microscopic analysis are two basic measurements required for the minimal characterization of reference materials. Phase contrast microscopy does not require staining to distinguish the "phase bright" dormant spores from phase dark spores, dark vegetative cells and clumps. When spores germinate they appear phase dark under phase contrast imaging (**5**). Germinated spores in a reference sample will soon die due to lack of nutrients. It is important in storing samples to prevent the premature germination of the spores. This standard practice includes the important steps for these measurements and includes guidance for advanced measurements. Additional guidance is given for advanced techniques to characterize spore suspensions that may be used to provide a higher level of characterized *Bacillus* spore reference samples.

5.4 The specific properties of the spores used for their intended application, such as susceptibility to disinfectant processes, should be determined in addition to the basic measurements covered in this practice. Additional information on the measurement of spore properties is located in the appendix.

6. Apparatus

6.1 *Pipettes,* fixed volume or adjustable. The performance of the pipettes should be checked to determine correct dispensing volume. (pipettors should be tested for proper performance frequently).

6.2 *Sterile Pipette Tips.*

6.3 *Vortex Mixer.*

6.4 *Incubator,* capable of maintaining 30 to 70 ± 2°C.

6.5 *Autoclave,* for preparing sterile media and sterilizing waste.

6.6 *Plate Spinner* (optional).

6.7 *Bunsen Burner or Alcohol Lamp* (optional).

6.8 *Sterile Glass or Sterile Plastic Disposable Spreader Rods.*

6.9 *Phase Contrast Microscope*—Low power (10 to 20×) and high power phase (40, 60, or 100×) objectives are preferred.

6.10 *Disposable Plastic (or Glass) Petri Dishes,* typically 100 mm in diameter and 15 mm deep, sterile.

6.11 *Dilution Tubes,* sterile.

6.12 *Glass Microscope Slides,* precleaned, typically 25 by 75 mm.

6.13 *Glass Coverslips.*

6.14 *Immersion Oil,* as recommended by microscope manufacturer suitable for objective used and coverslips.

7. Reagents

7.1 *Purity of Water*—Water used for preparation of solutions should be sterile and high purity; either reverse osmosis, de-ionized or distilled.

7.2 *Phosphate Buffered Saline (PBS)*—A typical composition is composed of 0.137 M NaCl, 0.0027 M KCl, 0.01 M sodium phosphate, pH 7.4. Other similar formulations may be used. The solution should be sterilized by autoclaving or filtration.

7.3 *Triton X100™ Stock Solution* (10 % vol./vol.), prepared in sterile water in a sterile container.

7.4 *Nutrient Agar Plates*—May be prepared in the laboratory or purchased. Plates should be stored and used within expiration date. Typically, laboratory prepared plates are stored at 4°C and used within 2 weeks. Specific media such as 5 % (vol./vol.) sheep blood agar plates may provide important colony morphology that can assist in the conformation of bacteria.

7.5 *Bleach,* freshly diluted, 10 % (vol./vol.). Confirm that the stock solution (commercial bleach, sodium hypochlorite) has not expired.

7.6 *Ethanol,* 70 % (vol./vol.), prepared in sterile water.

8. Hazards

8.1 Considerations for safe handling of spore suspensions. Some *Bacillus* spp. cause disease in human and animals. Prior to using these materials, the user must fully investigate the safety hazards associated with the particular *Bacillus* spp. and follow the appropriate safety guidelines. The correct training of personnel and the proper use of personal protective equipment (PPE) is essential. A good source for the information on laboratory safety is the publication "Biosafety in Microbiological and Biomedical laboratories" (**2**). Route of infection and infectious dose and toxin production impact potential for infection/toxicity.

8.2 Use of good microbiology practices is important for safely working with the pathogenic *Bacillus* species. International guidelines for microbiological safety should be followed when appropriate (**6**).

9. Sample Storage

9.1 Appropriate storage conditions are essential to preserve the properties of the spores. Preservation of spore viability (prevention of germination), sterility, and lack of clumping are the goals. Traditionally, spores have been stored in solutions of sterile water, 20 to 70 % (vol./vol.) ethanol or 1 % (wt./vol.) phenol in water at 4°C to prevent bacterial growth. Spores can be frozen, but the effect of freezing and thawing on the properties of the spores has to be determined.

9.2 It is essential to prevent premature germination of spores, clumping, and loss of spores during storage. One study used borosilicate glass vials with PTFE lined caps to store spores in a number of solutions including sterile deionized water, 20 % ethanol and 1 % phenol for periods up to several years at 4°C and found the spore viability for B. anthracis Sterne to be stable for over several years (**7**).

9.3 It is important that the storage condition for each particular source of spores should be investigated to determine the effect of storage on specific properties and viability. Traditionally spore preparations are considered to be high quality if they contain greater than 90 % phase-bright viable spores as determined in the following sections.

10. Procedure

10.1 *Spore Viability and Concentration by Growth on Agar-Containing Media:*

10.1.1 Several important factors should be considered when using *Bacillus* spore suspensions, including the selection of the solution used for dilution, the containers used for dilution, and any treatment to reduce potential aggregates (clumps) of spores present in the samples. The choice of dilution media and container should be selected to reduce losses of the spores due to adhesion to the surfaces of the container (and pipette surfaces) during dilution. A suitable solution for dilution of spore suspensions is PBS containing Triton X-100™ (0.05% vol./vol) (**8**). Other diluents and treatments can be used (**7-10**), but their suitability for the spores from a particular species of Bacillus bacteria should be determined.

10.1.2 Spore suspensions have to be adequately mixed immediately before dilution and sampling because the spores will settle in the bottom of the tubes. Vortex mixing the tubes for 30 s before removing a sample is an effective step to suspend the spores.

10.1.3 At least three replicates of the serial dilutions should be done on the spore samples. A typical dilution would be to vortex mix the stock suspension (30 s), immediately remove 0.1 mL and add this to 0.9 mL of the diluents in a new tube, resulting in a 10^{-1} dilution. Repeating this process from the 10^{-1} dilution tube results in a 10^{-2} dilution, and so on. It is important to use the dilutions immediately after preparation to prevent loss of spores due to adhesion to the walls of the container. The number of serial dilutions necessary will be dependent upon the concentration of the initial sample. The optimal dilution will be to result in 30 to 300 colonies when spread on a plate (see sections below). New sterile pipettes should be used for each dilution.

10.1.4 The spread plate method is commonly used to enumerate bacteria present in water (**3**). The diluted sample (as prepared above) is mixed and immediately dispensed (0.05 mL to 0.2 mL) onto the surface of the nutrient plate. It is desirable to have duplicate plates for each diluted sample. Sterile glass rods (L-shaped) or sterile plastic disposable spreaders are used to spread the inoculums evenly over the surface of the agar. A turntable device for spinning the plates speeds the process of spreading the inoculum on the agar surface of the plates. Plates are covered and are not moved until the liquid has been adsorbed. If the liquid is not absorbed, pre-dried plates may be used. The conditions for pre-drying the plates in an incubator overnight or under a sterile hood with the lid ajar should be determined for each type of agar media (**11**).

10.1.5 After spreading, the covers are replaced and the plates are placed in an incubator at the desired temperature (typically 30 to 37 ± 2°C). The temperature should be measured with a NIST-traceable thermometer.

10.1.6 A good source of the composition and performance of growth media is the Difco™ & BBL™ (**12**), Manual of Microbiological Culture Media, Remel™ catalog or the ASM Media Manual. Quality control of the growth media should be performed to ensure reliable results (**13**). The storage condition and shelf life of growth media, as recommended by the manufacturer, should be followed.

10.1.7 The plates are removed from the incubator, typically after 18 h, but the optimal time should be established depending upon the species, temperature and media used. The bacterial colonies are counted either manually, with an electronic counter or imaged with a camera. Only those plates having between 30 and 300 colonies should be used for determining the concentration of the spores.

10.1.8 The colony forming units (CFUs) in the sample are calculated by dividing the actual colonies on a plate by the volume applied to the plate. This result is divided by the dilution factor to calculate CFU/mL. The average CFU values and the standard deviations of the measurement should be reported. An example of the calculation is shown in the appendix.

10.1.9 *Low Concentrations of Spores*—It is anticipated that spore suspensions used as reference materials will be sufficiently concentrated for plate count determinations or visualization by phase contrast microscopy. However, with low concentrations of spores, samples can be concentrated by either centrifugation or filtration as used for detection of spores in water systems (**14**).

10.1.9.1 A short centrifugation, for example 12 000 × g for 5 min can be used. The spores can then be suspended in a smaller volume. Care must be taken to ensure this step has not resulted in losses or in the formation of spore clumps. Avoid excessive centrifugation or heating of samples during this step.

10.1.9.2 A large volume of spores can be concentrated on filters that can be directly placed on nutrient agar plates for enumeration of viable spores, such as the Standard Methods for the Examination of Water and Wastewater (**14**).

10.1.10 Controls should be included to ensure the plates, spreaders, pipettes and solutions are not contaminated. Comparison of colony morphology on the plates to known spore samples can be a valuable indicator of possible contamination. Blank control samples (buffers without any added spores) are spread on agar plates and treated in the same manner as the other samples. The presence of colonies on the blank controls indicates the presence of contamination or poor aseptic technique. Sterilized solutions and materials should be used to avoid contamination.

10.1.11 It is important to prevent carry-over between different samples and dilutions. The spreading rods must either be single use (that is, sterile disposable plastic rods) or glass rods that can be sterilized between dilutions. Glass rods can be