Improving Dispersion of Bacterial Endospores for Enumeration†

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Abstract

Precise enumeration of spores is crucial for accurate evaluation of spore survival in the presence of inactivating agents or extreme environmental conditions. Bacterial endospores tend to agglomerate, leading to low estimates of spore numbers. We have shown that addition of SDS to diluent used in counting bacterial endospores better disperses spores, leading to a ten-fold increase in counts. We attribute this effect to steric hindrance and electrostatic repulsion between micellar structures of SDS adsorbed at spore surfaces. We have also demonstrated that use of a low surface energy material (Teflon) improves spread-plate counts, an effect we attribute to lesser hold-up of liquid on Teflon versus glass.

Keywords: spore, enumeration, plating, agglomeration, surfactant, teflon

1. Introduction

Bacterial endospores have received considerable attention in the last few years. As a result of the anthrax attacks in 2001 (CDC, 2006), researchers are designing more potent and faster acting sporicidal agents. Survival of bacterial endospores is being investigated under extreme conditions of pressure, temperature and radiation, such as those encountered in outer space (Mastrapa et al., 2001; Moeller et al., 2008; Moeller et al., 2007; Nicholson et al., 2000). Precise enumeration of spores in a given sample is crucial for accurate evaluation of spore survival in the presence of inactivating agents or extreme environmental conditions. *Bacillus subtilis* and *Bacillus cereus* are common bacterial species employed for such investigations. Although *B. cereus* spores have an outer coat called exosporium, similar to *B. anthracis*, both *B. cereus* and *B. subtilis* (lacking exosporium) are employed as surrogates for *Bacillus anthracis* (Ivanova et al., 2003; Krishna V. et al., 2005; Krishna Vijay B., 2007; Nicholson et al., 2003; Read et al., 2003; Vohra et al., 2005; Vohra et al., 2006).

Plate counts are the primary means (gold standard) of enumerating viable spores and other techniques, such as optical microscopy or particle counting method, utilize the plate count method to verify the concentration of viable spores. Spores must be maintained in a dispersed state prior to plating in order to achieve accurate and reproducible counts. Bacterial endospores, particularly spores of *B. cereus*, are hydrophobic and thus tend to agglomerate (Peng et al., 2001; Ronner et al., 1990), leading to low estimates of spore numbers. Inaccurate counting can lead to misleading conclusions, particularly in spore inactivation studies. Agglomeration as a potential cause of inaccurate spore enumeration has been largely ignored. In the last ten years, more than 1000 papers have been published on inactivation of bacterial spores, however, only ten studies have considered agglomeration of spores during inactivation, and none has taken into account the agglomeration of spores during the enumeration step.

In the spread-plating method, which is one of the most widely used plate counting approaches, the spore suspension must be spread uniformly over the surface of the agar plates. The spreader material employed for spread-plating of spores is crucial in achieving uniform and reproducible counts (Koch, 1981). Use of a Teflon coating for the spreader was recommended by Koch because of its low affinity for bacterial cells (Koch, 1981). None of the literature reviewed mentions the type of spreader material.
We hypothesize that addition of surfactant to enhance dispersion of spores in suspension will significantly improve spore enumeration. Non-ionic surfactants, such as Tween80 are sometimes used for dispersion of bacteria. However, these are not suitable as dispersion aids for spores in inactivation studies as Tween80 can induce germination of spores thereby exposing the susceptible germinating spores to inactivating agents present along with spores (Parker et al., 1968). Similarly, cationic surfactants can induce germination and inactivate spores, and, thus, are not suitable as dispersion aids for spore enumeration (Pinzon-Arango et al., 2009). Anionic surfactants at lower concentrations are not known to induce germination or inactivate spores (Grund et al., 1982). Sodium dodecyl sulfate (SDS), an anionic surfactant used in washing of spores treated with lysozyme during purification, was employed as a model surfactant in the present study. Because of its anionic nature, SDS can reduce aggregation by enhancing electrostatic repulsion between spores. The presence of surfactant structures (e.g., micelles) on spore surfaces can act as a barrier to close approach of other particles, further limiting agglomeration. Teflon and glass spreaders were employed to investigate the effect of spreader material. Deionized water and phosphate buffered solution (PBS) were employed for spore storage and serial-dilution.

2. Materials and methods

2.1 Chemicals

Chemicals were obtained from Fisher Scientific (Hampton, NH), except as noted. Solutions and deionized water for washing bacterial suspensions were autoclaved before use. Lysozyme and phenylmethylsulfonyl fluoride (PMSF) solutions were prepared using autoclaved buffers. Lysozyme powder was stored at 4 °C and lysozyme solution was prepared from powder for each use. The PMSF powder was stored at 4 °C. PMSF solution was prepared by dissolving the powder in 2 to 3 mL (the minimum possible) ethanol. TEP buffer containing 50 mM Tris.Cl at pH 7.2, 10 mM EDTA, and 2 mM PMSF was prepared fresh for each use. Phosphate buffered saline (PBS) was prepared by combining 10.9 g of Na2HPO4, 3.2 g of NaH2PO4, 90 g of NaCl and deionized water to prepare 1 L of solution. The solution was autoclaved and diluted ten times prior to use.

2.2 Culturing of B. cereus spores

Culture media was prepared according to the American Society for Testing and Materials ASTM E2111-00 standard (ASTM, 2001) using Difco Columbia broth powder. Bacillus cereus (ATCC 2) colonies from Difco Tryptic soy agar plates were inoculated in 500 mL Erlenmeyer flasks containing 100 mL of 1/10th strength culture media fortified with 0.1 mM MnSO4.2H2O. The flasks were capped with foam plugs and wrapped in aluminum foil. The inoculated growth media was incubated for 72 h at 35 ± 2 °C on an orbital incubator-shaker (Model C24, New Brunswick Scientific, Edison, NJ) with a speed of 200 rev/min.

2.3 Purification of B. cereus spores

The B. cereus spores were purified by lysozyme method according to Xue and Nicholson (1996). Culture was centrifuged at 10,000 × g for 10 min at 4 °C and the pellet was resuspended in 20 mL of 1 M KCl/0.5 M NaCl. This suspension was centrifuged and the pellet was resuspended in 20 mL of 50 mM Tris.Cl (pH 7.2) containing 50 μg/mL lysozyme and incubated at 37 °C for 60 minutes. The suspension was then washed with, respectively, 1 M NaCl, deionized water, 0.05 % (w/v) SDS, TEP buffer, and deionized water (three times). The washed suspension was heat shocked at 80 ± 2 °C for 15 min, followed immediately by cooling to 4 °C. Spores were stored at 4 °C in deionized water for no more than one week. The purity (proportion of spores relative to the total number of spores and cells) of the final suspension was 99 %.

2.4 Counting

The spore suspension was serially diluted (ten-fold) and aliquots of the three highest dilutions (either dilution numbers 2, 3 and 4 or 3, 4 and 5) were plated in triplicate on Tryptic soy agar in 100 × 25 mm Petri dishes by the spread-plate technique. The plates were then incubated at 37 °C for 12–16 hours and then the colonies were counted. Counts between 30 and 300 were used for data analysis (Koch, 1981).

2.5 Statistical tests

Hypothesis testing for the significance of differences between two means was based on two-tailed Student’s t test (Sokal et al., 1997). Post-hoc testing of the significance of the differences between three or more means was carried out by Tukey’s test (Berthouex et al., 1994).

2.6 Surface tension measurements

Surface tension was used to estimate the critical micelle concentration of SDS in phosphate buffered saline. Surface tension was determined by the Wilhelmy plate technique,
which measures the maximum force required to pull a vertical platinum plate from solution at the liquid-air interface. Measurements were made at SDS concentrations of 0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 5.00 and 10.00 mM in PBS at 24 ± 2 °C.

2.7 Particle counting

Spores suspended in PBS with and without SDS were counted with a Multisizer™ 3 Coulter Counter® (Beckman Coulter, Inc., Fullerton, CA) using a 5 μm orifice. The spores were serially diluted to obtain a final concentration of 10^5 CFU ml⁻¹ in the sample holder.

2.8 Zeta potential

Zeta potential of B. cereus spores in deionized water (pH = 5.8) was determined with a Brookhaven ZetaPlus (Brookhaven Instruments Corporation, Holtsville, NY).

3. Results and discussion

3.1 Effect of suspending media and surfactant

Average colony counts of Bacillus cereus spores plated in triplicate, with DI water and PBS as diluents, are given in Fig. 1. The colony counts with DI water were two times higher than the counts obtained with PBS. Since the starting material (bacterial spore suspension) was the same in the various trials, an increase in spore counts is indicative of a lesser degree of agglomeration. Thus the lower counts obtained with PBS versus distilled water suggest a higher extent of spore agglomeration in the PBS. B. cereus spores have very low zeta potential (−11 mV in DI water) compared to bacteria (−40 mV) (Li et al., 2004; Peng, Tsai and Chou, 2001) and, thus, are more susceptible to changes in ionic strength of diluent. The high ionic strength (0.2 M) of PBS can effectively screen the net negative charge and aid in agglomeration of spores.

Sodium dodecyl sulfate (SDS) was added to PBS for the purpose of improving the dispersion of spores. Fig. 2 shows the plot of surface tension as a function of SDS concentration. The critical micelle concentration (the concentration at which surfactant molecules self-assemble into micelles) for SDS in phosphate buffered saline was found to be 1 mM, which is close to the reported value of 0.83 mM at 0.2 M NaCl (Rosen, 2004). The CMC for SDS in DI water is 8 mM (Rosen, 2004). Thus, the beneficial effect of SDS is achieved in PBS with a much lower concentration of SDS (e.g., 1 mM) than would be required for DI water. The effect of SDS concentration on colony counts with PBS as diluent is shown in Fig. 3. Significant improvement is obtained at one-half the critical micelle concentration (CMC). Further improvement was observed at a concentration equal to twice the CMC. Counts achieved in PBS with 2 mM SDS were much higher than...
counts achieved in DI water with 2 mM SDS (Fig. 1). Enhanced dispersion with SDS addition was verified by particle counts (Fig. 4). In the presence of SDS, a peak in the concentration of B. cereus spores, as indicated by the measured count frequency, occurred in the range of 1–1.5 µm. Without SDS, counts were generally lower and no peak was observed. Since the length of B. cereus spores is in the range of 0.95–1.5 µm, it is apparent that much higher numbers of individual spores are found in the presence of SDS. Since the outer protein coats of spores have positively and negatively charged sites as well as hydrophobic sites, it is possible that micelles or hemi-micelles of an anionic surfactant such as SDS can adsorb on spore surfaces and inhibit agglomeration by steric hindrance and electrostatic repulsion. The observation of higher spore counts in the presence of SDS supports this conjecture.

3.2 Effect of spreader material

Compounding errors in counting due to incomplete spore dispersion is the potential for liquid hold-up by the spreader. A thinner film of spore suspension will adhere to Teflon than to glass because of Teflon’s lower surface energy (20 vs. 47 mN m⁻¹). Thus, with Teflon, there will be a smaller hold-up volume of spore suspension remaining on the spreader when it is removed from the agar surface. Indeed, significantly higher counts and more consistent counts were obtained with a Teflon spreader compared to a glass spreader, with or without SDS present (Table 1). However, addition of SDS to the diluent increased the spore counts ten-fold with either spreader material. The combination of a Teflon spreader and SDS amended diluent provided the highest counts.

Table 1  Effect of spreader material on spore counts

<table>
<thead>
<tr>
<th>Spread Material</th>
<th>PBS</th>
<th>PBS + 2 mM SDS</th>
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<tbody>
<tr>
<td>Glass</td>
<td>2.4 × 10⁵ ± 18%</td>
<td>5.2 × 10⁶ ± 20%</td>
</tr>
<tr>
<td>Teflon</td>
<td>5.0 × 10⁵ ± 17%</td>
<td>7.5 × 10⁶ ± 5%</td>
</tr>
</tbody>
</table>

* Means were significantly different at α = 0.05; one-tailed t-test.

4. Conclusions

We have shown that agglomeration is a significant problem in enumeration of B. cereus spores. An anionic surfactant was successful in dispersing spores and increasing the spore counts. The adherence of liquid to the spreader is another source of error in spread-plate counting. This problem can be ameliorated by use of a low surface energy material, such as Teflon, for the spreader.

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### Author's short biography

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Dr. Vijay Krishna is an Assistant Professor of Molecular Medicine in the Department of Biomedical Engineering at the Lerner Research Institute, Cleveland Clinic. His current research interests include carbon nanostructures, nanomedicine and light-nanomaterial interactions for biomedical applications. Dr. Krishna received his B.E. in Chemical Engineering from Bangalore University and Ph.D. in Materials Science and Engineering from University of Florida. He continued as a Postdoctoral Associate at the Particle Engineering Research Center at the University of Florida. Dr. Krishna has 19 peer-reviewed publications and 11 patents.
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Dr. Brij M. Moudgil is a Distinguished Professor of Materials Science and Engineering at the University of Florida. His current research interests are in particulate materials based systems for enhanced performance in bioimaging, nanomedicine, photocatalytic degradation of hazardous microbes, polymer and surfactant adsorption, dispersion and aggregation of fine particles and nanotoxicity. Dr. Moudgil received his B.E from the Indian Institute of Science, Bangalore, India and his M.S and Eng.Sc.D degrees from Columbia University, New York. He has published more than 400 technical papers and has been awarded 14 patents. He is a member of the U.S National Academy of Engineering.