Processing Bacillus Vegetative Cells and Spores Using Pressure Cycling Technology (PCT)

Bioterrorism remains an ever-present threat to the world. Rapid detection systems are critical to civilian security and to reducing casualties on the battlefield. Most research activities to date in counter-bioterrorism have focused on the development of critical amplification, identification, and detection systems. However, sample preparation is an equally critical aspect of rapid identification and detection of bioterror agents, but the enhancement of such systems lags well behind the development of amplification, identification, and detection systems.

Of particular threat to humans is *Bacillus anthracis*, which can be acquired by inhaling *B. anthracis* spores from animal hair and wool or by deliberate exposure to weapon grade preparations of spores. However, *B. anthracis* is only one of a number of potential bio-terror agents. Here we describe the use of the non-infectious species *Bacillus subtilis,* as a model organism for the development of methods for extracting DNA from vegetative cells and spores.

Bacillus spores are particularly resilient and often remain viable despite chemical and physical treatments. The extraction of DNA from spores for detection is even more challenging. Current methods of lysis designed for the extraction of DNA from bacterial spores include chemical and physical methods, such as bead beading. However, these methods have significant limitations and draw-backs. To increase the safety, speed, simplicity, and efficiency of DNA extraction from biological samples, including spores, Pressure BioSciences has developed several methods using a combination of chemicals and the bio-physical method of cycled pressure (pressure cycling technology or PCT) to extract DNA from such challenging organisms.

Pressure Cycling Technology (PCT)

PCT uses alternating cycles of high and ambient pressures to induce cell lysis. Cell suspensions or a solid sample, such as bacterial spores in a matrix, are placed in specially designed, single-use processing containers (PULSE Tubes) and are then subjected to alternating cycles of high (up to 35,000 PSI) and ambient pressures in a pressure-generating instrument (Barocycler®) – together the PCT Sample Preparation System (PCT SPS). Maximum and minimum pressures, the time at each pressure level, and the number of cycles are defined using a programmable logic controller. The reaction chamber of the Barocycler instrument can be temperature controlled using a peripheral circulating water bath. Safety features in the design of the PCT SPS significantly reduce risk of operator exposure to pathogens and prevent cross-contamination of samples. The PCT SPS offers a safer, more efficient method for DNA extraction than other methods in use today.

Methods

A series of experiments was conducted to demonstrate the effectiveness of PCT on inactivation and lysis of Bacillus sp. vegetative cells and spores.

Processing B. subtilis Vegetative Cells

High concentration *B. subtilis* was prepared in liquid media according to the protocol described in Reference 1. PCR amplification procedures were as described in Reference 2.

In one set of experiments, vegetative *B. subtilis* cells were treated using PCT at 35 kpsi for ten one-minute cycles. Non-treated control samples and PCT-treated samples were plated on TSA plates at 32°C overnight and examined for the formation of colonies (Fig.1).

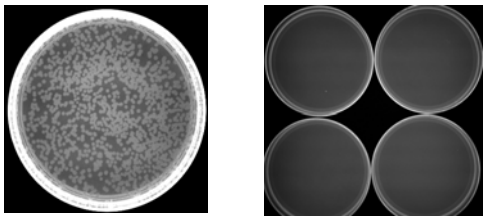

Figure 1. *TSA Agar Plates of B. subtilis Cultures A) Non-treated negative control B) PCT- treated.*

In other experiments, *B. subtilis* cells, started from a single colony, were either (a) cultivated directly in PULSE Tubes at 32°C for 5 hrs, (b) 1.3 mL of the seed culture was transferred into each PULSE Tube and incubated at 32°C overnight, or (c) 1.3 mL of cells that were cultured in a flask overnight, were transferred to PULSE Tubes for PCT treatment. All samples were processed at 35,000 psi for ten one-minute cycles. The processed samples and the negative controls (samples that were not PCT treated) were first centrifuged at 13,000 rpm for 3 min. Supernatants of these samples were then amplified directly in PCR reactions and visualized in an agarose gel (Fig. 2).

In similar experiments, spores were used to inoculate PULSE Tubes containing TSB media. The spores were allowed to form vegetative cells for various periods of time prior to PCT-treatment and PCR amplification (Figure 3).

Processing B. subtilis Spores

B. subtilis spores were prepared, pelleted, and suspended in ATL buffer with 1% Triton (Qiagen) or saturated with Guanidinium HCl supplemented with 1% CHAPS 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate, 1% Triton and 1% SDS. Samples were then PCT-treated at 35 kpsi for five one-minute cycles at 22°C. Other samples were prepared by bead beating for five 30-second treatments with 30- second cooling breaks between treatments. Negative controls were prepared using the same samples and buffers, but these were not pressure treated. After pressure treatment, DNA was purified using Qiagen DNeasy Mini kit from 250 µL clarified supernatants. PCT extracts were split into two sets. One was heated at 70°C for 10 minutes prior to adding ethanol in the purification according to the kit procedure. The other set was not heated. Final DNA products were dissolved in 100 µL buffer AE and visualized in an agarose gel.

Results and Discussion

Figure 1 demonstrates that pressure cycling technology (PCT) is capable of inactivating the vegetative form of *B. subtilis* under relatively mild conditions. However, in similar experiments with spores, stronger reagents and mechanical treatments were not able to inactivate all spores regardless of the sample preparation method used. These data suggest that samples that are contaminated with *B. anthracis* spores may remain infectious and thus must be considered hazardous even after substantial sample preparation, regardless of the method chosen. However, sample preparation systems such as the PCT SPS, which include additional safety features inherent in their design, and may therefore be preferable to many other existing extraction methods.

Figure 2 shows that the PULSE Tube may potentially be used as a culture tube to enable Bacillus spores to transform to the vegetative state.

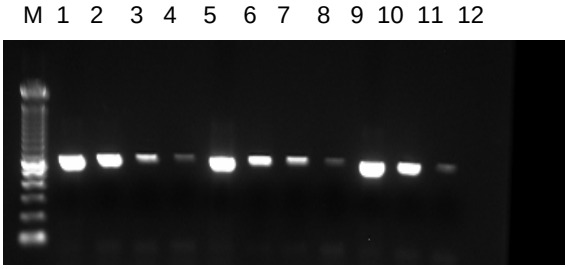

Figure 2. *PCR examination of B. subtilis samples cultured and PCT treated in Pulse Tubes. B. subtilis cells were directly cultivated in PULSE Tubes (Lanes1-4) at 32°C for 5 hours at dilutions of 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}; in flasks (Lane 5 to 8); or in a flask first followed by transfer to PULSE Tubes (Lanes 9-12). All samples were then subjected to 10 cycles of PCT treatment at 13,000 rpm for 3 minutes, and crude supernatants were subjected to PCR amplification.*

In other experiments, spores were allowed to transform to the vegetative state and replicate in the PULSE Tube resulting in a "biological" amplification of target DNA (Figure 3).

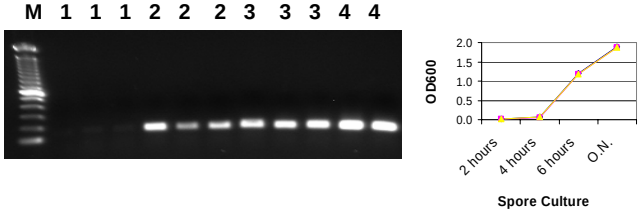

Figure 3. *Improvement in sensitivity after growth of B. subtilis spores in PULSE Tubes. Lanes 1-4 correspond to PCR products from Time 0, 2, 4, and 6 hrs respectively at RT.*

Figure 4 shows that PCT in combination with the proper buffer selection is capable of releasing DNA from Bacillus spores. Yields are similar to the bead beating method.

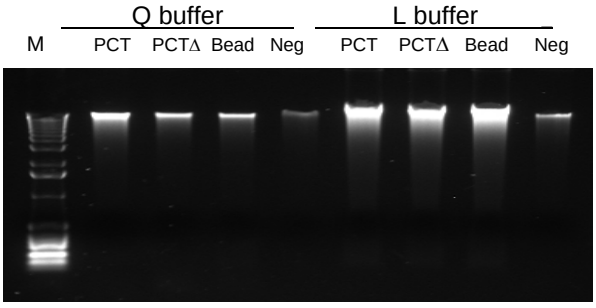

Figure 4. *Agarose gel comparing genomic DNA extracted from B. subtilis spores by PCT or Bead Beating with two different extraction buffers (Q and L).*

Conclusion

Data show that PCT is capable of inactivating *B. subtilis* vegetative cells. In addition, the PCT SPS in combination with chaotropic-based lysis buffers, such as Guanidinium HCl (?), has been shown to release significant amounts of DNA suitable for amplification by PCR. In our experiments, PCT is comparable in sensitivity to bead beating methods of preparation. However, many factors, such as purification methods, probe design, PCR conditions (endpoint vs. real-time), and the quality of the spore preparation will affect the absolute sensitivity. Regardless of the sample preparation method that is employed, one must assume that some portion of spores may remain viable. Therefore, selection of a sample preparation method should also include safety features inherent in the design of the system.

The PCT SPS is a closed system that uses a single-use, disposable PULSE Tube. Since the PULSE Tube can be used as a collection, storage, transportation, and growth tube, it is superior to other sample preparation systems. Other experiments (data not shown) have demonstrated that the PCT SPS is capable of directly processing contaminated paper or cloth without additional preparation. The PCT SPS can be used with secondary and tertiary containment protocols during processing. Reducing the risk of cross-contamination improves accuracy while providing a significant increase in user safety. The Barocycler automates the sample extraction process, which makes the PCT SPS safe, fast, efficient, and reproducible.

References

[1] "Molecular and Biological Methods for Bacillus" Harwood and Cutting Editors, John Wiley and Sons Publishing, pp. 414-418

[2] Makino S, Cheun H. Application of the real-time PCR for the detection of airborne microbial pathogens in reference to the anthrax spores. *J. Microbiol Methods* 2003 May; 53(2):141-7

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