Chemical Engineering Journal 279 (2015) 578–588

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Inactivation of bacterial spores subjected to sub-second thermal stress

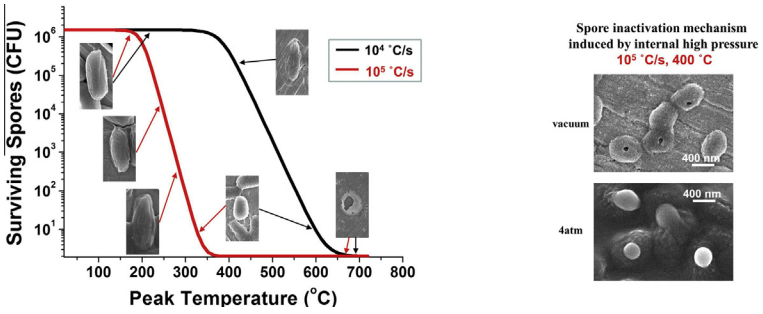
W. Zhou [a], M.W. Orr [c], G. Jian [b], S.K. Watt [c], V.T. Lee [c,*], M.R. Zachariah [a,b,*]

[a] Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, MD 20742, USA
[b] Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA
[c] Department of Cell Biology & Molecular Genetics, University of Maryland, College Park, MD 20742, USA

HIGHLIGHTS

- A new approach to quantitatively apply high rate heat stress is developed.
- Spore inactivation with temperature followed a sigmoidal behavior with higher heating rates improving the neutralization efficiency.
- The peak temperature and not heating time was responsible for spore inactivation at $\sim 10^4$ °C/s.
- Viability reduction was mainly due to DNA damage at $\sim 10^4$ °C/s.
- Spore inactivation was mainly due to internal pressurization at $\sim 10^5$ °C/s.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 February 2015
Received in revised form 28 April 2015
Accepted 5 May 2015
Available online 14 May 2015

Keywords:
Bacterial spores
Inactivation
Ultrafast heating
Killing mechanism

ABSTRACT

Rapid heat pulse is the primary method for neutralizing large quantities of spores. Characterizing heat inactivation on a millisecond time scale has been limited by the ability to apply ultrafast, uniform heating to spores. Using our system for immobilization of spores on metal surfaces, bacterial spores were subjected to high temperatures (200–800 °C) and heating rates ($\sim 10^3$ °C/s to $\sim 10^5$ °C/s). Spore inactivation increased with temperature and fit a sigmoid response. We observed the critical peak temperature (T_c) which caused a 2-fold reduction in spore viability was 382 °C and 199 °C for heating rates of $\sim 10^4$ °C/s and $\sim 10^5$ °C/s, respectively. Repetitive heating to the same peak temperature had little effect on viability. In contrast, stepwise heating to elevated peak temperatures inactivated spores in a manner similar to a single pulse heating to the same peak temperature. These results indicate that the maximum temperature rather than the overall heating time is primarily responsible for spore neutralization at $\sim 10^4$ °C/s heating rate. The mechanism of spore inactivation was further investigated at two heating rates ($\sim 10^4$ °C/s and $\sim 10^5$ °C/s). Viability reduction was mainly due to DNA damage at the heating rate of $\sim 10^4$ °C/s as mutant strains defective for *sspA sspB* and *recA* were more sensitive to heat than the wide-type strains. At the higher heating rate ($\sim 10^5$ °C/s), spore inactivation was correlated with physical damage from ultrafast vapor pressurization inside spores. This new approach of pulse heating generates a temperature, time, and kill relationship for *Bacillus* spores at sub-second timescales.

* Corresponding authors.
E-mail addresses: vtlee@umd.edu (V.T. Lee), mrz@umd.edu (M.R. Zachariah).

http://dx.doi.org/10.1016/j.cej.2015.05.021
1385-8947/

1. Introduction

Bacterial spores are a dormant cell type that are highly resistant to heat, moisture, pressurization, radiation and biocidal stresses [1–3]. Concerns regarding bio-terrorism [4] and possibly other public health risks [5,6] require the urgent development of methods for large scale inactivation of bacterial spores. The use of thermal destruction is a straightforward and convenient approach that has been investigated for the inactivation of spores in the liquid phase [7–16]. Conventional thermal inactivation methods require time periods up to minutes or even hours and are limited by a maximum temperature of 150 °C [8–10,11,13]. Maintaining these temperatures for the required time periods is unfeasible for large quantities of spores. Thus, stand-off neutralization methods which can sustain higher temperatures (>200 °C) for short periods of time (sub-seconds) are being considered, and these new temperature–time–kill histories of spore inactivation require evaluation. Aerosol methods have recently been employed to evaluate thermal and chemical kills of spores at extreme heating conditions [17–25]. These approaches have the advantage of isotropic and rapid heat transfer to individual spores, as well as controllable shorter heating times (milliseconds to seconds) and higher heating temperatures (>200 °C) [17–25]. However, there existed a temperature distribution within a population of spores in these studies, which allows for calculating the average resident time and temperature of the entire spore population, but not for individual spores. These studies evaluated the effect of peak temperature on spore viability. However, the effects of heating and cooling rates on spore inactivation were not discussed.

Precise measurements of heating rate and heating history can be achieved by an alternative method that heats spores immobilized on metal supports. Previous studies have shown that organic or inorganic nano/microparticles deposited on metal surfaces could be heated to a wide range of surface temperature (from ~100 °C to ~1800 °C) at a rate as high as $\sim 10^6$ °C/s [26–31]. In those studies, the heating time could be controlled with millisecond precision and the heating rate could be accurately tuned from $\sim 10^3$ °C/s to $\sim 10^6$ °C/s, allowing the application of uniform temperature to a deposited particle layer up to 5 μm thick [29] which exceeds the dimensions of a monolayer of bacterial spores. This approach allows accurate application of a uniform high temperature over millisecond time scales for individual spores, which will aid in determining the temperature–time–kill relationship of spores at high temperatures and sub-second timescales.

The mechanism of spore inactivation has been primarily studied in conventional heating schemes. Major mechanisms of spore inactivation occurred through damage to DNA and proteins [3]. Evidence supporting killing by DNA damage includes several studies that found mutants depleted in the *sspA sspB* genes (coding for α/β-type small, acid-soluble proteins (SASP)) and *recA* genes (coding for RecA) were sensitized to mutagenesis and inactivation under dry heating schemes [32–36]. In the dormant spores, SASP bind genomic DNA [32,33] to prevent depurination and strand breakage thereby confer resistance to dry heat and ultraviolet irradiation [34,35]. RecA participates in recombination repair to remove DNA lesions caused by dry heat [36]. In contrast, denaturation of some key proteins such as metabolic enzymes was found as the mechanism of spore killing in wet heat [8,37]. However, the specific proteins to which denaturation induces spore inactivation have not been identified [38]. A second mechanism of inactivation is through permeabilization of the spore membrane, cortex and coat (encoded by genes like *cotE*), as supported by several lines of evidence [3,39–44]. Damage to spore compartments after heating has been shown with compartment-specific staining techniques [45,46]. While the spore coat was identified as the primary barrier to oxidizing agents (hydrogen peroxide, hypochlorite, ozone, etc.) [47–49], the spore cortex plays a major role in maintaining spore resistance to heat [3,39]. Failure of the cortex structure leads to spore inactivation by two mechanisms. The first is the release of dipicolinic acid (DPA) associated with DNA damage during dry heating [40,41] and protein denaturation during wet heating [38]. The second is through rehydration of the spore protoplast [39,42]. Rehydration of the core will induce a concomitant reduction in spore heat resistance by disrupting SASP–DNA interaction and permitting protein denaturation [43,44]. Through these studies, the temperature–time–kill relationships for spores from several species have been characterized under conventional heating schemes [8–11,13]. However, the exact mechanism of spore inactivation during rapid heating, which is closer to conditions seen under combustion, is poorly understood. Recent studies of bacterial spores heated in hot air show that high-temperature gas induced severe damage to the spore core [50], and the extent of inactivation was attributed to the DNA damage [22].

In this work, we present the effect of fast heating pulses ($\sim 10^4$ °C/s and $\sim 10^5$ °C/s) on surface-immobilized spores as a complimentary approach to the aerosol studies. Our heating scheme possesses the advantages of precise measurement of heating time and rates, as well as uniform temporal temperature for individual spores. Using this heating scheme, we determined the effect of peak heating temperature and heat rate by assessing spore viability and morphology. The heating histories of spores were also investigated through repetitive exposures as a possible factor influencing spore inactivation. To investigate the mechanism of killing, spores carrying *sspA sspB*, *recA* or *cotE* mutations were tested to determine whether known mechanisms of spore inactivation contribute to spore viability in our heating scheme. Using these results, we propose a model for the thermal destruction of bacterial spores in the heating rates of $\sim 10^4$ °C/s and $\sim 10^5$ °C/s.

2. Materials and methods

2.1. Spore attachment on platinum wires

Bacillus subtilis (Bs) (ATCC#6051) were sporulated in Difco Sporulation Medium (DSM) at 30 °C for 48 h. The 250 ml of DSM included 2 g Bacto nutrient broth, 2.5 ml 10% KCl, 0.375 ml 1 M NaOH and 2.5 ml 1.2% $MgSO_4 \cdot 7H_2O$. The spore concentration was enumerated by plating to be 8×10^9 colony-forming units per milliliter (CFU/ml). The purity of spores was found more than 99%. For analysis of mechanism of killing, four isogenic Bs spore strains, namely, a wild-type strain (PS533) [36], a Δ*cotE* mutant strain lacking coat (PS3328) [48], a Δ*sspA* Δ*sspB* mutant strain lacking DNA protection mechanism (PS578) [32], and a Δ*recA* mutant strain lacking DNA repair mechanism (PS2318b) [36] were generously provided by Dr. Peter Setlow (University of Connecticut). A platinum (Pt) wire with a diameter of 76.8 μm (Omega Engineering, Inc.) was used to immobilize spores. An in-house spore deposition cell was manufactured for coating the wire with Bs spores electrophoretically (Fig. S1). By controlling the biased deposition voltage, pulse frequency, and charging time (from a 6340 sub-femtoamp remote sourcemeter, Keithley), a uniform monolayer of spores in the central region of the wire (~1 cm) could be obtained (Fig. 1A). The detailed information of the spore deposition cell and charging conditions can be found in our previous work [51].

2.2. Wire heating test

To subject spores to a defined thermal history, the spore coated Pt wire was connected to an in-house built power source, working