Chemical Engineering Science 144 (2016) 39–47

Contents lists available at ScienceDirect

Chemical Engineering Science

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

Synergistic effects of ultrafast heating and gaseous chlorine on the neutralization of bacterial spores

Wenbo Zhou [a], Mona W. Orr [b], Vincent T. Lee [b,*], Michael R. Zachariah [a,**]

[a] Department of Chemical and Biomolecular Engineering, and Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA

[b] Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742, USA

HIGHLIGHTS

- Spore neutralization was improved by the synergistic effect of rapid heat and Cl_2.
- Spore coat detached from the underlying core at >450 °C with Cl_2 at 10^5 °C/s.
- Humidified Cl_2 caused more viability reduction than dry Cl_2.
- The majority of Cl content was located in the shed spore coat.

ARTICLE INFO

Article history:
Received 11 August 2015
Received in revised form
10 December 2015
Accepted 1 January 2016
Available online 21 January 2016

Keywords:
Bacterial spores
Neutralization
Ultrafast heating
Chlorine
Hydrogen chloride

ABSTRACT

Improving the neutralization of bacterial spores is of paramount importance for the bioterrorism defeat. In this study, we investigate the synergism between rapid heating ($\sim10^4$ °C/s to $\sim10^5$ °C/s) and chlorine gas in the neutralization of *Bacillus thuringiensis* (Bt) spores – a close relative of *Bacillus anthracis* (Ba), which is a known biowarfare agent. Bt spores were heated in a gas chamber with defined concentrations of Cl_2 gas and relative humidity (RH). The critical peak temperature (T_c) of spores, which corresponds to 50% reduction in viability, was decreased from 405 °C when heated at $\sim10^4$ °C/s in air to 250 °C when heated at the same rate in 100 ppm Cl_2. SEM results show no obvious difference between the morphologies of spores heated in air or in Cl_2 at $\sim10^4$ °C/s. These results indicate that Cl_2 gas acts in synergy with high temperatures (>300 °C) to neutralize Bt spores. Similarly, the T_c for Bt spores heated at the faster rate of $\sim10^5$ °C/s was reduced from 230 °C when heated in air to 175 °C when heated in 100 ppm Cl_2. At $\sim10^5$ °C/s, the treatment of Cl_2 did not alter spore morphology at temperatures below 300 °C. At temperatures above 450 °C with Cl_2, the spore coat detached from the underlying core. The effect of Cl_2 was further examined by changing the RH of Cl_2 gas. The results show that highly humidified Cl_2 (RH=100%) reduced T_c by 170 °C and 70 °C at $\sim10^4$ °C/s and $\sim10^5$ °C/s, respectively, as compared to dry Cl_2 (RH=0%). Energy dispersive spectrometric (EDS) results demonstrate that Cl_2 on the spore increased with elevated peak temperature, with the majority of the Cl located in the shed spore coat. This study indicates that the major mechanism of spore neutralization by the synergism of Cl_2 and rapid heat is chlorine reacting with the spore surface.

1. Introduction

Bacterial spores have raised serious concerns in the military biodefense (Nadasi et al., 2007; Gilbert and Duchaine, 2009; Kummer and Thiel, 2008). Neutralization of spores is a significant challenge since they are much more resistant than their vegetative counterparts to a variety of external stresses such as UV irradiation, extreme pH values, chemicals, and temperature extremes (Gould, 2006; Nicholson et al., 2000; Setlow, 2006). Studies have shown that spore longevity and resilience is correlated to physical features of spores, including a tight proteinaceous spore coat that inhibits chemicals penetration, low water content in the spore core to reduce metabolism, as well as the production of proteins for DNA stabilization (e.g. α/β-type small acid soluble proteins (SASPs)) (Setlow, 2006). One of the primary strategies for spore neutralization is to expose them to autoclaving heat (120–150 °C) for minutes to hours (Conesa et al., 2003; Couvert et al., 2005; Coleman et al., 2007); however, this approach is not appropriate for the large-scale neutralization of spores. To further improve the killing

* Corresponding author. Tel.: +1 301 405 9397; fax: +1 301 314 1248.
** Corresponding author. Tel.: +1 301 405 4311.
E-mail addresses: vtlee@umd.edu (V.T. Lee), mrz@umd.edu (M.R. Zachariah).

http://dx.doi.org/10.1016/j.ces.2016.01.019
0009-2509/

efficacy, aerosol based techniques are being developed to rapidly inactivate airborne bacterial spores by rapid heating (> 200 °C within a timescale of a second) (Gates et al., 2010, 2011; Grinshpun et al., 2010a, 2010b; Johansson et al., 2011; Jung et al., 2009; Lee and Lee, 2006). At this timescale, more than 3-logs reduction in spore population can be achieved when the peak temperature ranges from 200 to 400 °C. An analogous approach of spore inactivation by heat has been tested using heat generated from exothermic reactions of energetic materials such as aluminum-based thermites (Lee et al., 2013). This approach is able to produce even higher peak temperatures (> 2200 °C) over a shorter period (~ 0.1 s), and leads to a 7-log reduction of spore viability. Although these methods are capable of neutralize spores, an accurate and quantitative relationship of time–temperature–kill for spores is not available due to the variability in temperature distribution and the resident exposure time of these heating schemes. Nevertheless, a precise time–temperature–kill relationship is needed for predicting and ensuring a successful outcome of large-scale neutralization events. In order to improve the accuracy of measurements of the temperature history on spores, an alternative approach has been developed by heating spores deposited on conductive surfaces (Childs, 2001) that allows measurement of the transient temperature using the standard electric resistance–temperature relationships (Childs, 2001). Since the transient temperature of immobilized spores can approximate that on the immobilizing surface (Zhou et al., 2015), the time–temperature–kill relationship for spores can be accurately measured. Results showed that a 6-log reduction in spore counts could be achieved by rapidly heating spores to 600 °C within 50 ms at a heating rate of $\sim 10^4$ °C/s. The neutralization mechanism was likely due to DNA damage as mutants in *sspA sspB* are sensitized for killing (Setlow, 2006). Faster heating rates ($\sim 10^5$ °C/s) also improved spore neutralization, which was associated with increased structural destruction of spore coat through increased pressure of vaporization (Zhou et al., 2015).

In addition to heat, another commonly used disinfection procedure for spores utilizes biocidal chemicals (McDonnell and Russell, 1999). Commonly used biocides (antibiotics, detergents, alcohol) have little effect on spore viability (Fraise, 2011). In contrast, strong oxidation agents, such as chlorine, iodine, sulfur, silver, and compounds containing these elements, have demonstrated efficacy in spore inactivation (McDonnell and Russell, 1999). Among these sporicides, Cl_2 is one of the few agents that are gaseous at room temperature. The main advantage of Cl_2 over other aqueous sporicides is that gas provides greater coverage, thus facilitating the neutralization of both airborne and surface-associated spores. Cl_2 can directly chlorinate functional groups on macromolecules in cells to damage proteins, nucleic acids and lipids (Coombs and Danielli, 1959; Deborde and von Gunten, 2008). In addition, Cl_2 can react with water to form hypochlorous acid (HOCl) and hydrogen chloride (HCl). Both compounds can also react with the spore to inactivate them (DeQueiroz and Day, 2008; Young and Setlow, 2003).

The performance of Cl_2 depends on two characteristic factors: concentration ("C") and inactivation time ("T"). The US Enivronmental Protection Agency and the water treatment industry has set the units of Cl_2 concentration in parts per million (ppm) and the inactivation time in minutes (Rose et al., 2005). In general, the "CT" product is a constant for spores of a specific *Bacillus* strain required to achieve a defined reduction of viability (Brazis et al., 1958). Table 1S presents some documented "CT" results for different *Bacillus* spores (DeQueiroz and Day, 2008; Rose et al., 2005; Brazis et al., 1958; Rice et al., 2005; Fair et al., 1947; Hosni et al., 2009; Szabo and Minamyer, 2014; Kreske et al., 2006; Venczel et al., 1997; Johnson et al., 2003; Son et al., 2004; Perez et al., 2005; John et al., 2005; Thorn et al., 2013). The "CT" product for a 4-log reduction in spore viability is $\sim 3 \times 10^4$ ppm min (~ 100 mg min/l) for most of *Bacillus* spores when the Cl_2 concentration is below 3×10^3 ppm (Rose et al., 2005; Brazis et al., 1958; Rice et al., 2005; Fair et al., 1947; Hosni et al., 2009). At much higher Cl_2 concentrations (e.g. 7×10^6 ppm), this "CT" value is significantly larger than $\sim 3 \times 10^4$ ppm min, and the minimum exposure time is 5 min (DeQueiroz and Day, 2008; Szabo and Minamyer, 2014; Kreske et al., 2006). In order to improve the neutralization efficiency at exposure times under a second, which according to the "CT" rule would require concentrations of Cl_2 $> 10^8$ ppm (close to that of pure Cl_2 liquid). The use of these concentrations of Cl_2 would be impractical as a method to safely neutralize spores (http://emergency.cdc.gov/agent/chlorine/basics/facts.asp). To meet the guidelines set by the US Food and Drug Administration (FDA) for food and drinking water processing (Code of Federal Regulations Title 21 Part 173/178) (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=173■■■■;■■■■ http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=178), new approaches are in development to combine heat with 100–2000 ppm Cl_2.

One such potent, more environment-friendly and safer approach is to synergize the neutralization of spores by Cl_2 with heat. Xu et al. (2008) studied the inactivation of *Bacillus* spores by Cl_2 (~ 1000 ppm) under high-temperature short-time pasteurization conditions (~ 80 °C, ~ 1 min), and found a viability reduction of 6-logs. Further tests using a higher temperature of 120 °C resulted in inactivation of spores by 6-logs within 16 s, whereas it took > 30 min to achieve viability reduction of 6-logs by employing either Cl_2 gas (1000 ppm) (Table S1) or heat (120 °C) (Conesa et al., 2003; Couvert et al., 2005; Coleman et al., 2007). Based on these results, higher temperatures (> 200 °C), and diluted Cl_2 gas (< 2000 ppm) synergistically inactivated bacterial spores.

In this work, we investigated the synergistic effects of ultrafast heating and Cl_2 gas on the inactivation of *Bacillus thuringiensis* (Bt) spores. Bt spores, while closely related to *Bacillus anthracis* (Ba) spores that are considered as a serious bioterrorist weapon, is not pathogenic to humans. Both Bt and Ba spores were reported to have similar sensitivity to biocides (Sagripanti et al., 2007), so we expect the results in this study can be utilized for the neutralization of Ba spores in the future. For these studies, Bt spores were electrophoretically deposited onto a fine Pt wire (Zhou et al., 2015). By tuning the heat pulse time (2 ms and 50 ms) and peak temperature (up to ~ 1200 °C) for the Pt wire, the heating rate ($\sim 10^4$ °C/s and $\sim 10^5$ °C/s) and time-resolved temperature for individual spores were measured (Zhou et al., 2015). Using this thermal approach, we evaluated the effect of 100 ppm (0.3 mg/l) Cl_2, a concentration below the FDA safety guidelines, in combination with different peak temperatures and heating rates, on the neutralization of spores. Spore viability and morphology were assessed after these treatments by determining the viable number of colony forming units (CFU) and scanning electron microscopy (SEM). To investigate the neutralization mechanism of Cl_2, spores were also heated in Cl_2 gas with different relative humidities, to see the roles of Cl_2 and its hydrolytic products (hydrogen chloride (HCl) and hypochlorous acid (HOCl)). Energy dispersive X-ray spectroscopy (EDS) was employed to determine the elemental changes of Cl and carbon (C) in spores.

2. Materials and methods

2.1. Spore attachment on platinum wires

Bt spores 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 to be more than 99%. Bt spores were electrophoretically immobilized onto a sterilized platinum (Pt) wire with a diameter of 76.8 μm (Omega Engineering, Inc.). The