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Research article

# Inactivation of *Geobacillus stearothermophilus* spores by alkaline hydrolysis applied to medical waste treatment

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ABSTRACT

Although alkaline hydrolysis treatment emerges as an alternative disinfection/sterilization method for medical waste, information on its effects on the inactivation of biological indicators is scarce. The effects of alkaline treatment on the resistance of *Geobacillus stearothermophilus* spores were investigated and the influence of temperature (80 °C, 100 °C and 110 °C) and NaOH concentration was evaluated. In addition, spore inactivation in the presence of animal tissues and discarded medical components, used as surrogate of medical waste, was also assessed. The effectiveness of the alkaline treatment was carried out by determination of survival curves and D-values. No significant differences were seen in D-values obtained at 80 °C and 100 °C for NaOH concentrations of 0.5 M and 0.75 M. The D-values obtained at 110 °C (2.3–0.5 min) were approximately 3 times lower than those at 100 °C (8.8–1.6 min). Independent of the presence of animal tissues and discarded medical components, 6 log10 reduction times varied between 66 and 5 min at 100 °C-0.1 M NaOH and 110 °C-1 M NaOH, respectively. The alkaline treatment may be used in future as a disinfection or sterilization alternative method for contaminated waste.



## 1. Introduction

A large number of methods are available to inactivate microorganisms. Most of them use the same fundamental principle of heat, chemicals, irradiation or combinations of these. Several methods are currently used for the sterilization, defined as a process that destroys all forms of life including dormant. These methods include plasma, vapour-phase hydrogen peroxide, ozone, chloride dioxide, autoclaving, ethylene oxide and radiation. The selection of the method depends on the type of material being treated as well as the intended purpose. For instance, the last three methods are the most widely used for the sterilization of medical instruments. Each of these methods has advantages and disadvantages. Autoclaving is usually employed to kill bacteria, viable spores including endospores and virus in heat resistant materials. At 121 °C or higher, sterilization is achieved. When temperatures below 121 °C are used a disinfection process occurs, which may kill vegetative forms of microorganisms, such as pathogens or other harmful organisms but do not inactivate bacterial endospores (Russell, 2001). Autoclaving is extremely time-consuming and is not adequate to treat heat sensitive materials. Exposure to ethylene oxide is highly efficient due to its penetrative properties. Therefore, it is considered one of the most suitable sterilization processes for thermo sensitive materials. However, ethylene oxide is extremely toxic and presents risks associated with handling a flammable (Mendes et al., 2007). Radiation by gamma rays or electron beam are also very effective sterilization methods, but can affect product integrity and can degrade polymers and rubbers. Additionally, their utilization requires high capital investment (Haji-Saeid et al., 2007). Plasma technology has been studied as an alternative to conventional sterilization methods (Kylián et al., 2006; Yardimci and Setlow, 2010). This method has some advantages over others, such as low energy consumption, absence of residuals and toxic emissions, safety and low capital and operational costs (Yardimci and Setlow, 2010). Nevertheless, it has a particular limitation, namely its incompatibility with some polymeric materials (Lerouge et al., 2002).

Sterilization processes are not only necessary for high added-value materials. Indeed, tonnes of medical waste are produced per year (Diaz et al., 2008; Lee and Huffman, 1996) and must be treated to eliminate the infectious potential prior to disposal.

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Autoclaving and incineration are the main processes used for treating medical waste, the last being the oldest and, until now, the most used (Lee and Huffman, 1996; Sukandar et al., 2006). However, this process demands high investment and exploration costs and it is not appropriate to treat small quantities of medical wastes. In this context, it is essential to develop effective low cost alternative sterilization processes.

Various microorganisms, including pathogens, produce dormant forms, which permit their survival under stress conditions, such as high temperature, irradiation or chemical damage. Amongst these structures, the endospores, herein further designated as spores, produced by some low G + C Gram-positive bacteria, are the most resistant to harsh conditions. Several spore traits have been described to be involved on resistance against physical and chemical antimicrobial agents. The low water content in the spore core seems to be the most important factor of a spore wet heat resistance. Indeed, the wet heat resistance correlates negatively with the core water content (Setlow, 2006). The high core mineralization also confers wet heat resistance; ions such as $Ca^{2+}$ ensure a higher wet heat protection than $Mg^{2+}$, $Mn^{2+}$, $Na^{+}$ and $K^{+}$. Another essential factor to the spore resistance is the high quantity of small acid-soluble spore proteins (SASPs) that protect the spore DNA by its saturation with α/β-type SASP and DNA repair systems (Leggett et al., 2012; Setlow, 2006).

*Geobacillus stearothermophilus* comprise low G + C Gram-positive, thermophilic non-pathogenic bacteria, and their spores are one of the most heat and chemical agents resistant. Indeed, the low water content in the core and the intrinsic thermostability of proteins confers to spores of thermophilic species a higher resistant to wet heat than to those of mesophiles (Guizelini et al., 2012). Therefore, the spores of this organism are often used as a biological indicator to assess the effectiveness of sterilization methods (López et al., 1997; Watanabe et al., 2003; Wood et al., 2010).

This study reports the alkaline treatment as a disinfection and a sterilization alternative methods for waste contaminated with infectious agents. The successful inactivation of a Creutzfeldt–Jajob disease (CDJ) agent (Taguchi et al., 1991), the inactivation of 22A strain of scrapie agent (Taylor et al., 1997), the prion decontamination (McDonnell et al., 2013; Murphy et al., 2009) and inactivation of potentially infectious agents including virus, bacteria, fungi and protozoa (Kaye et al., 1998; Murphy et al., 2007; Neyens et al., 2003; Dixon et al., 2012) have been proved.

In the present study the effect of alkaline treatment on the degree of *G. stearothermophilus* spores inactivation, in terms of decimal reduction times (D-value), at three temperatures (80 °C, 100 °C and 110 °C) and different sodium hydroxide concentrations, was assessed. In addition, dipicolinic acid (DPA) released from endospores after the alkaline treatment was detected by the terbium dipicolinate fluorescence method.

## 2. Material and methods

### 2.1. Preparation of G. stearothermophilus spores

Strain *G. stearothermophilus* $22^{T}$ was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *G. stearothermophilus* was grown in Nutrient Agar (Liofilchem) at 55 °C for 4 days. After incubation, the biomass was scraped from the agar surface and washed with sterile distilled water. The resulting suspension was incubated at 80 °C for 15 min. After cooling down, the suspension was centrifuged at 1000 × g for 30 min at 5 °C. The supernatant was decanted, and the biomass was washed in chilled sterile distilled water and re-centrifuged. This step was repeated twice. After re-suspension in water, the suspension was incubated at 37 °C for 60 min in the presence of lysozyme (100 μg/mL) for peptidoglycan breakdown. After washing with sterile distilled water for three times, and centrifugation at 1000 × g for 20 min at 5 °C, the suspension was incubated with sodium dodecyl sulphate (SDS) at 2.5% and incubated at 60 °C for 15 min, to increase the membrane fragmentation. After, the spores were washed with sterile distilled water for three times. Confirmation of the integrity of cells and spores after each step was carried out through transmission electron microscopy analysis (Fig. 1). The final suspension of spores was serially diluted with sterile distilled water to obtain approximately $10^{7}$ colony-forming units per mL (cfu/mL) and stored at 4 °C.

### 2.2. Alkaline treatment

The experiments were carried out in a Parr batch reactor with a titanium vessel of 450 mL capacity under temperature control. Five millilitres of spore suspension at $10^{7}$ cfu/mL was mixed with 45 mL of NaOH solution at different concentrations (0.1 M, 0.25 M, 0.5 M, 0.75 M or 1 M). The reactor was heated at temperatures of 80 °C, 100 °C or 110 °C with heating rates of 5 °C/min. When the temperature stabilized, samples of 1.5–2 mL were taken, at regular time intervals up to 30 min. A control was made by heating the spore suspension at 100 °C without NaOH.

To evaluate the behaviour of spores in the presence of material usually present in medical waste, experiments with animal tissues (pork meat and bone) and a mix of discarded medical components (cotton, diapers, tubes for transfusion, surgical gloves, examination gloves, adhesives, surgical masks, bag collectors for urine, serum bottles and syringes) were performed. Except for cotton, the animal tissues were cut in fragments of approximately 1 $cm^{2}$ and all the assays were carried out using samples with 1 g of each component. The experiments performed with those materials were carried out at the same conditions used in their absence. Approximately 10 g of animal tissues or discarded medical components was added to the spore suspension ($10^{7}$ cfu/mL) with 50 mL of 0.5 M NaOH solution.

### 2.3. Incubation and survival counts

The number of surviving spores was determined by the viable plate count method. Samples of heated spore suspensions (1.5–2 mL) were cooled in ice-water and neutralized with an HCl solution to pH 7. Samples were serially diluted in sterile saline solution (0.85% NaCl, w:v) and 0.1 mL were spread on triplicate nutrient agar plates and incubated at 55 °C for 24 h, 48 h, 72 h, 96 h and 120 h. It was verified an increase in the cell counts over time, stabilizing at 96 h. Thus, the D-values were calculated using data obtained after 96 h of incubation. A positive control consisting on the enumeration of the total cell counts of the spore suspension used in each assay was performed in parallel.

### 2.4. Fluorimetric detection of DPA

The DPA released by a $10^{6}$ cfu/mL spore suspension after autoclaving at 121 °C for 30 min and after the hydrolysis at 110 °C, with 1 M NaOH was determined through a fluorimetric method, as previously described (Navarro et al., 2008). Briefly, a 1000 μL aliquot of suspension was added into 1 cm quartz cuvette with 40 μL of 10 nM $TbCl_3$ and 800 μL water distilled. The photoluminescence was measured at 270 nm excitation and 546 nm emission wavelengths. A calibration curve was prepared with DPA (2,6 pyridinedicarboxylic) concentrations ranging from 0 up to 10 nM. As control, a standard DPA solution at 10 nM was quantified after the aforementioned autoclaving and alkaline treatments. Four independent replicates were carried out for each condition.