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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 ºC, 100 ºC and 110 ºC) 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 ºC and 100 ºC for NaOH concentrations of 0.5 M and 0.75 M. The D-values obtained at 110 ºC (2.3-0.5 min) were approximately 3 times lower than those at 100 ºC (8.8e1.6 min). Independent of the presence of animal tissues and discarded medical components, 6 log10 reduction times varied between 66 and 5 min at 100 ºC-0.1 M NaOH and 110 ºC-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 endo-spores and virus in heat resistant materials. At 121 ^{o}C or higher, sterilization is achieved. When temperatures below 121 ^{o}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 (Kyliả n 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.

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). How- ever, 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 a/b-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 ^{o}C, 100 ^{o}C and 110 ^{o}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 ^{o}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 ^{o}C for 15 min. After cooling down, the suspension was centrifuged at 1000 g for 30 min at 5 ^{o}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 ^{o}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 (1.5e2 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 auto- claving at 121 ^oC for 30 min and after the hydrolysis at 110 ^oC, 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 mL water distilled. The photo- luminescence 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.

2.5. *Transmission electron microscopy*

Bacterial and spore suspensions were fixed for 4 h with 2.5% glutaraldehyde and 4% formaldehyde (obtained from hydrolysis of paraformaldehyde) diluted in 0.1 M cacodylate buffer (pH 7.2), post-fixed overnight with 2% OsO_4 in cacodylate buffer, stained in bloc with 1% uranyl acetate, dehydrated with ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate before being observed in a JEOL 100CXII transmission electron microscope.

3. Results

Preliminary assays with vegetative cells of *G stearothermophilus* (Fig. 1A) demonstrated that they were very sensitive to alkaline solutions, since 0.1 M NaOH at 100 ^oC killed 99.9999% of the initial 10^6 cells/mL after 30 min of contact (data not shown). Under the same conditions, 99.9% of the *G. stearothermophilus* spores were inactivated. In opposition, the spores were not inactivated at 100 ^oC in the absence of NaOH (Fig. 2). Spore morphology was not affected by treatment with lysozyme (Fig. 1B) or SDS (Fig. 1C).

The survival curves, obtained when the *G. stearothermophilus* spores were exposed to alkaline conditions, exhibited biphasic curves with a slope tailing, as shown in Figs. 2 and 3. There are several models that describe the inactivation of microorganisms (Chick, 1908; Cole et al., 1993; Kamau et al., 1990). Cerf (1977) proposed a model for populations constituted by two-fraction with a constant inactivation rate for each fraction. In this model it is assumed that inactivation of both fractions is independent and irreversible, each following first order kinetics (Equation (1)).

$$\frac{N(t)}{N_0} = fe^{-k_1 t} + (1-f)e^{-k_2 t} \qquad (1)$$

where $N(t)/N_0$ is the proportion of surviving spores, t is the exposition time (min), k_1 and k_2 ($k_1 > k_2 \geq 0$) are the death rate constants for first fraction and second fraction, respectively, f and $(1-f)$ are the initial proportion in first fraction and second fraction, respectively, and e is the Naperian base. The first and second fractions describe the death of the less and the more resistant spores, respectively (Xiong et al., 1999).

Given the biphasic behaviour of the survival curves of the *G. stearothermophilus* spores, the D-values, at specified conditions, were determined by estimating the parameter values of the Cerf's model. Table 1 reports the D-values, 6 log10, f, k_1, and k_2. The differences between the D-values at 80 °C and 100 °C were not significant for NaOH concentrations of 0.5 M (1.9 and 1.6 min, respectively) and 0.75 M (1.0 and 0.9 min, respectively); the 6 log10 reduction times were also similar. Given that both incubation at 80 °C and 100 °C are currently used to isolate spores of low G C content Gram positive bacteria (Gerhardt, 1994), at these two temperatures spore inactivation was solely due to the presence of the alkaline solution. Indeed, no spore inactivation was observed in the controls performed at 100 °C in the absence of NaOH, as shown in Fig. 2. At 100 °C, 6 log10 reductions varied between 66.1 and 19.7 min in the presence of 0.1 and 0.75 M NaOH, respectively.

As expected, for each temperature tested, the calculated D- values decreased with the increase of the NaOH concentration. At 100 °C, the lowest D-value (0.9 min), obtained with 0.75 M NaOH, was about ten times lower than that obtained with 0.1 M NaOH (8.8 min).

On the other hand, for each NaOH concentration tested, the calculated D-values decreased with the increase of temperature. For NaOH concentrations of 0.1 M, 0.25 M and 0.5 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). The combined effect of high temperature (110 °C) and NaOH (1 M) led to the complete inactivation of spores (6 log10 reduction) after 5 min.

To confirm spores inactivation, DPA released after alkaline treatment (110 °C, 1 M NaOH, 30 min) was quantified, and compared to that released after autoclaving (121 °C, 30 min). The concentration of DPA after autoclaving (1.8 nM) was approximately 3.5 times higher than that quantified after the alkaline treatment (0.5 nM) (Table 2). Given this unexpected result, the effect of temperature and NaOH on the DPA determination was carried out, using a 10 nM standard solution of this compound. It was verified that the presence of NaOH interfere with the DPA quantification, since after the alkaline treatment the concentration of this organic acid was about 3 times lower than that after heating at 110 °C for 30 min (Table 2). Confirmation of spores destruction after the alkaline treatment was given

by TEM analysis. No spores were observed after the alkaline treatment (Fig. 1D).

Given the importance of medical waste sterilization, the behaviour of spores in the presence of materials usually present in medical waste was assessed at 110 ^{o}C and 0.5 M NaOH. In first minutes (1-2 min), the rate of spores inactivation in the presence of animal tissues and discarded medical components was similar to that in the absence of materials, as shown in Fig. 3. However, after that period, there was a higher heat and alkaline resistance of those spores comparably to the ones solely in NaOH solution. Such differences can be explained by diffusion mechanism that occurred with sodium hydroxide and materials. In addition, NaOH consumption in hydrolysis of the materials occurred. Indeed, under the conditions tested, the animal tissues were almost destroyed. Nevertheless, the time required for the complete inactivation of spores in the presence of animal tissues and discarded medical components (25 min and 26 min, respectively) was not much longer than that needed in their absence (24 min).

4. Discussion

The survival curves of *G. stearothermophilus* spores after being subjected to alkaline treatment are typical of a mixture of two fractions or sub-populations with different resistance to stressful conditions, such as heat (Abraham et al., 1990). This difference in heat resistance has been attributed to different physiological states in the spore population (Iciek et al., 2006). A dormant spore transits to a vegetative cell by activation, germination and outgrowth. The activation is a reversible process; only when the germination phase starts the spore can no longer return to its dormant state (Leggett et al., 2012). Hence, spore suspensions may contain sub- populations of activated and dormant ones. Spores in the activated state are described as more sensitive to stressful conditions than in the dormant state. Thus, in the present study the spores were inactivated in two stages: the first corresponds, most probably, to the inactivation of the less resistant spores and the second of the more resistant ones.

The decline observed in spore heat resistance, when the temperature of 110 ^{o}C was used, can be explained by an increase in the core water content. Although the mechanism of spore inactivation by wet heat is not entirely clear yet, it is partially due to the rupture of the spore inner membrane permeability barrier, which causes an increase in the core water content (Setlow, 2006). The spores inactivation by alkaline treatment seems to involve the removal of alkali-soluble coat proteins with consequent inactivation of the lytic enzymes essential for cortex hydrolysis and spore germination (Duncan et al., 1972). Treatment efficiency can be proved by the release of DPA to the suspension after the alkaline treatment and TEM observations.

It has been previously reported that inactivation of *G. stearothermophilus* at low temperatures (<100 ^{o}C) can be achieved using chemicals agents (Mazzola et al., 2003; Rogers et al., 2007), high-pressure carbon dioxide (Watanabe et al., 2003) and supercritical carbon dioxide with added hydrogen peroxide (Hemmer et al.,

2007) but the time required to inactivate spores is high. The D-values found in literature for inactivation assays carried out at temperature 100 °C were higher than those obtained in this work (Table S1), except when using high pressure treatments (Patazca et al., 2006). The D-values herein obtained at 100 °C were even lower than those found in studies using thermal inactivation at temperatures above 100 °C. Except in the experiments carried out with 0.1 M NaOH, the highest D-value obtained was 2.3 min (Table 1). In contrast, at 120 °C, López et al. (1997) reported D- values ranging from 1.32 to 2.84 min, while at 121 °C, Feeherry et al. (1987) and Guizelini et al. (2012) reported D-values from 1.3 to 5.4 min. Nevertheless, the time required to complete inactivation of *G. stearothermophilus* spores in the present study was probably, different from those obtained in abovementioned studies. Indeed, at 121 °C the thermal inactivation of spores generally follows a first order linear kinetics while under alkaline treatment, as described above, inactivation curves were non-linear.

Some authors (Murphy et al., 2009; Thacker, 2004) estimated the costs for the alkaline hydrolysis treatment. Thacker (2004) indicates costs of \$320 ton^{-1}, including those with steam, water, electricity, chemicals, labor, sanitary sewer and maintenance and repair. Similar values (\$260-\$310 ton^{-1}) were obtained by Murphy et al. (2009) using alkaline hydrolysis to dispose of animal tissues and carcasses during their study on prion inactivation. These costs do not include the initial capital investment. The sterilization conditions, i.e., relation temperature/time herein obtained were less aggressive than those described on previous studies. Hence, it can be argued that operation costs may be lower than reported before. However, further scale-up studies are needed to assess the detailed costs under the herein described conditions.

5. Conclusions

The results herein obtained confirm previous reports on the effectiveness of alkaline on the treatment of biologically contaminated waste. Low temperature values (110 °C), NaOH concentration (1 M) and time (5 min) were needed to achieve sterilization. The time required for total inactivation of spores in the presence of the tested animal tissues and discarded medical components, identical to those commonly found in medical waste, was similar to that obtained in their absence. The disadvantage of this treatment is the production of an effluent with high alkalinity, which adds to the process one additional neutralization step before discharge.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jenvman.2015.06.045.

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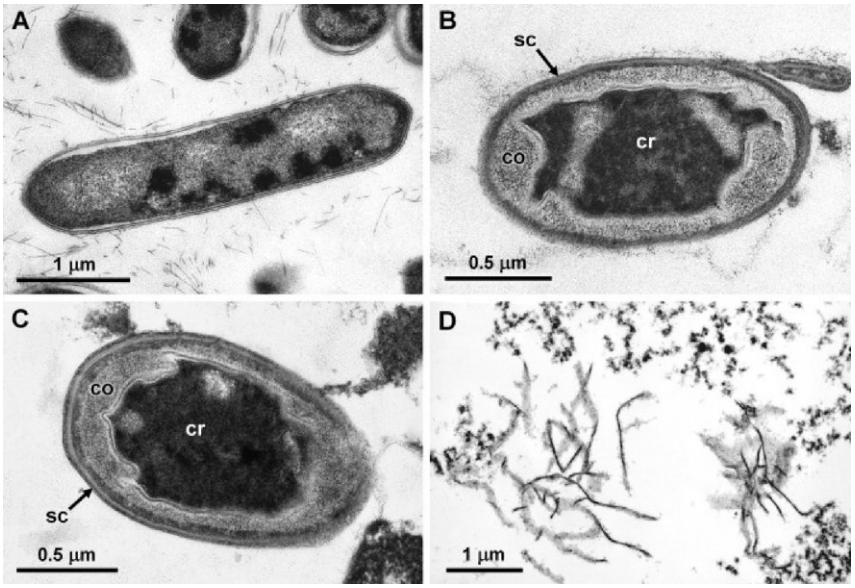

Fig. 1. TEM images of *Geobacillus stearothermophilus*. Vegetative cells suspension (A), spores suspension after addition of lysozyme (B), after addition of SDS (C) and spore debris after alkaline treatment (D). *sc*, spore coat; *co*, cortex; *cr,* core.

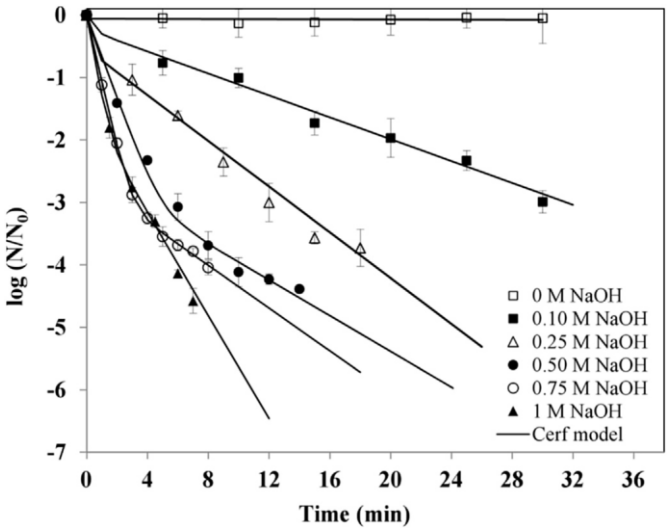

Fig. 2. Survival curves for the *Geobacillus stearothermophilus* exposed to alkaline treatment at 100 ºC with various NaOH concentrations. Vertical bars represent standard deviations of the means.

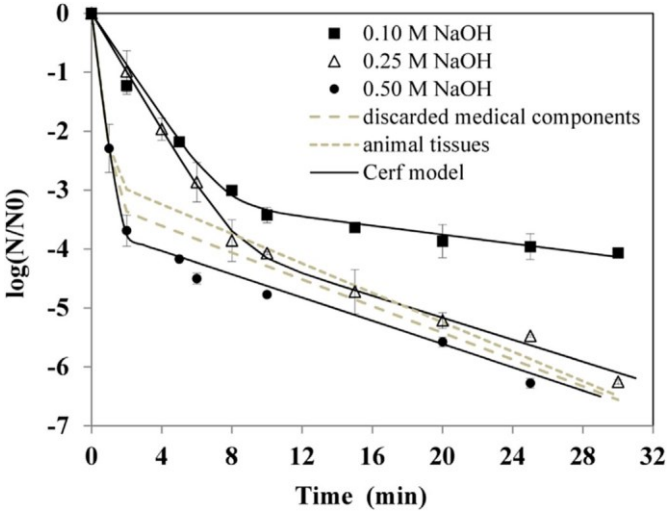

Fig. 3. Survival curves for the *Geobacillus stearothermophilus* exposed to alkaline treatment at 110 ºC with various NaOH concentrations. Vertical bars represent standard deviations of the means.

Table 1

Decimal time reduction (D-value), 6 log10, estimates of the model parameters and standard derivation values for the Cerf model. Data presented are the mean of three in- dependent experiences with standard deviation.

Conditions		D-value (min)	6 log10 (min)	f	k_1 (min^{-1})	k_2 (min^{-1})
Temperature (°C)	NaOH (M)					
80	0.50	1.9 ± 0.2	28.4 ± 2.7	0.9919 ± 0.0219	1.2168 ± 0.0777	0.3176 ± 0.1179
80	0.75	1.0 ± 0.0	20.2 ± 0.0	0.9993 ± 0.0000	2.3189 ± 0.2117	0.3909 ± 0.0280
100	0.10	8.8 ± 0.8	66.1 ± 1.5	0.4144 ± 0.0866	3.0538 ± 0.0000	0.2010 ± 0.0029
100	0.25	2.5 ± 0.2	29.8 ± 3.6	0.7170 ± 0.0577	5.9338 ± 0.1111	0.4218 ± 0.0531
100	0.50	1.6 ± 0.2	24.3 ± 3.2	0.9967 ± 0.0026	1.5322 ± 0.2923	0.3296 ± 0.1792
100	0.75	0.9 ± 0.0	19.7 ± 1.1	0.9977 ± 0.0008	2.4692 ± 0.0604	0.3940 ± 0.0343
100	1.00	0.8 ± 0.0	10.8 ± 0.1	0.9691 ± 0.0369	3.1831 ± 0.1160	0.9500 ± 0.0168
110	0.10	2.3 ± 0.1	40.8 ± 6.0	0.9990 ± 0.0029	1.0075 ± 0.0752	0.0872 ± 0.0806
110	0.25	1.8 ± 0.3	29.0 ± 2.2	0.9993 ± 0.0005	1.1392 ± 0.0287	0.2148 ± 0.0197
110	0.50	0.5 ± 0.0	24.0 ± 0.1	0.9998 ± 0.0004	5.2480 ± 0.1350	0.2274 ± 0.0523

Table 2

Concentration of 10 nM standard DPA, and DPA released from endospores after autoclaving and alkaline treatment.

Conditions	DPA released (nM)
DPA standard (110 °C, 30 min)	9.8 ± 0.0
DPA standard (110 °C, 1 M NaOH, 30 min)	3.3 ± 0.2
Autoclaving (121 °C, 30 min)	1.8 ± 0.1
Alkaline treatment (110 °C, 1 M NaOH, 30 min)	0.5 ± 0.1