Bacteria inactivation by pulsed electron beam

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Pulsed electron beam is a promising physical technology which enables to sterilize products or packaging. However, the mechanisms involved in bacteria inactivation are still not fully known. In this work, we have investigated by using electron microscopy the effects of pulsed electron beam on the bacteria-envelope. The results reveal a high efficiency of bacteria inactivation but curiously, without any structural modification in the cell-envelope. The explanation of the killing effect remains pending.

Keywords—pulsed electron beam, Bacillus pumilus, inactivation, SEM, TEM

I. INTRODUCTION

Food and pharmaceutical industries use different types of sterilization methods: chemical (hydrogen peroxide…), thermal or physical (gamma ray, X-ray or electron beam) technologies. However, these solutions are time consuming, cost effective and sometimes harmful to health (e.g. hydrogen peroxide). Within context, the low energy pulsed electron beam is promising and allows sterilization without using chemical compounds and can be an IN-LINE process. The equipment presented delivers more than 1 kGy in a 10 ns pulse width at a repetition rate of 100 Hz that leads to very high dose rate and makes sterilization possible at less than 25 kGy in the millisecond range. Electron beam was extracted from a cathode and went through an anode, consisted of metallic foil and called the extraction window.

Here, we first present the efficiency of pulsed electron beam on bacteria (vegetative form and spore) to select the best model for the study. Then, we investigated if an adaptive mechanism of spores to electron beam is present. Finally, we tried to explain the mechanisms involved in the killing effect.

II. MATERIALS AND METHODS

A. Strain selection and growth conditions

The first strain selected is *Bacillus atrophaeus* ATCC 9372 which is the reference for food industries. The second one is *Bacillus pumilus* ATCC 27142. This strain is the reference for ionization treatment [1] [2].

These strains can be found under vegetative (growth phase) and spore (dormant) forms. The last form is resistant to environmental stresses.

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Vegetative cells were cultivated in Luria broth (LB) medium overnight at 37°C. Cells were centrifuged during 5 minutes at 6 000g and the pellet was resuspended in sterile deionized water. The final concentration of bacteria was 10 times more important than the initial solution.

Spore cells had grown after stationary phase, during 5 days at 37°C. The medium was the following: 8g/L of Difco Nutrient Broth (Difco), 100 mM of $MgSO_4$, 10% of KCl, 1M of NaOH, 1M of $Ca(NO_3)_2$, 10 mM of $MnCl_2$ and 1mM of $FeSO_4$. To eliminate vegetative forms, the solution was heat-shocked at 80°C during 20 minutes. Spores were recovered by centrifugation (5 minutes at 10 000g). Spores were collected on sterile deionized water in order to have 10 times more bacteria than the cultivated solution.

B. Treatment

The voltage and current of the generator used for this experiment were 250 kV and 5 kA, respectively. The pulse duration was 10 ns. For the experiment, two parameters were studied: the pulsed repetition frequency (from 5 to 100 Hz) and the distance from the extraction window (from 2 cm to 9 cm).

C. Determination of inactivation rate

- For treatment on agar, different concentrations of bacteria were spread on Petri dishes. The dishes were treated with the pulsed electron beam and incubated overnight at 37°C. The colonies were counted and the inactivation rate was calculated thanks to the equation (1).
- For dry treatment, 10 µL of solution were put on empty Petri dished and dried under laminar flow. Following the treatment, spots were resuspended in sterile distilled water and different dilutions were applied and spread on Plate Count Agar (PCA) medium overnight at 37°C. The equation (1) was used to determine the inactivation rate.

$$IR = \log_{10}(N0/N) \quad (1)$$

IR	inactivation rate
N0	initial microbial concentration
N	number of survivors after the treatment

D. *Resistance evaluation*

After an exposition to electron beam at sub-lethal dose, bacteria can acquire a better resistance to the treatment for the same dose [1] [3] [4].

To determine if there is a modification of resistance with our technology, two solutions of *Bacillus pumilus* spores with 10^{10} spores/mL were prepared and a spot of 10µL was deposed on Petri dishes. The first solution called Wild Type (WT) was the stock solution. It was the reference for the efficiency. The other one was cultivated from bacteria which was treated under lethal dose x time(s). After the irradiation, spots were recovered as described on *II. C.* part for 'dry treatment' and survival strain were counted. The decontamination rates were compared. The two Petri dishes were placed at the same time under the electron beam.

The treatment applied for each sample was 10 pulses à 7 centimeters from the extraction window, with a pulsed repetition frequency of 100 Hz.

E. *Scanning Electron microscopy and Transmission Electron Microscopy observation*

P.R. Chalise and S.E. Fiester shown pores formation due to electron beam treatment. A high density of current could induce the formation of irreversible pores around the bacteria [5] [6]. Electron microscopy was used to observe if this phenomenon appears with pulsed electron beam. Bacteria were fixed with 2% glutaraldehyde in 0.1M Sorensen phosphate buffer pH 7.2 just after the recovered. The samples were prepared by the CMEAB platform, Toulouse (France).

For Scanning Electron microscopy (SEM), images were acquired with an electron microscope Quanta^{TM} 250 FEG (FEI, USA) at an accelerating voltage of 10 kV.

For Transmission Electron Microscopy (TEM), sections had a thickness of 70 nm and were placed on a grid. Images were visualized with HT 7700 at 80 kV (Hitachi, USA).

III. RESULTS

A. *Selection of reference strain*

We made treatment on agar placed at 7 centimeters and with a pulsed repetition frequency of 100 Hz to select the most resistant strain. Vegetative form of *Bacillus atrophaeus* was irradiated with 2 pulses. In this condition, the logarithmic reduction is more than 5.7. For the spore form of this bacteria, we need 10 pulses to obtain 4.47 $\log_{10}$ reductions. Consequently, as described in the literature, spore form of bacteria is more resistant than vegetative form. Indeed, the different layers which composed the spore, the presence of a core with condensed DNA, the low concentration of water and other parameters explain the faculty of this form to survive in extreme conditions [7] [8] [9].

The experiment was made on *Bacillus pumilus* spores, which are the reference for irradiation technologies. This strain is effectively more resistant than *Bacillus atrophaeus* spores because after a treatment with 10 pulses, the logarithmic reduction obtained was 4.1 $\log_{10}$.

This is the strain selected for the rest of the study.

B. *Parameters influence*

- The first parameter studied was the pulsed repetition frequency (Hz). For these experiments, *Bacillus pumilus* spores were spotted on empty Petri dishes and placed under the extraction window at 7 centimeters. Inactivation was calculated by colony counting and with the equation (1). The results revealed that for 50Hz and 100Hz, the decontamination rate was the same. However, for 5Hz, the efficiency was better (Fig. 1). These phenomena can be explained because the switch of the generator is self-triggered, consequently, for low frequency, the parameters are different than parameters for high repetition frequency. Consequently, we cannot conclude that the frequency has an influence on the mortality rate of this strain. Because this equipment can be used for industrial applications, the maximum repetition frequency was chosen for the next experiments.
- The second parameter was the distance from the extraction window. For these experiments, *Bacillus pumilus* spores were spotted on empty Petri dishes and placed under the extraction window at 2, 4, 7 and 9centimeters. The repetition frequency was 100Hz. Inactivation was calculated by colony counting and with the equation (1). On Fig. 2, the graph revealed a better efficiency when bacteria were placed close to the extraction window. These phenomena can be explained because of electron beam interaction with molecules present in the air and their path was modified. The interaction with molecules decreased also energy of electrons. These two phenomena induced a decreased of the dose with the distance.

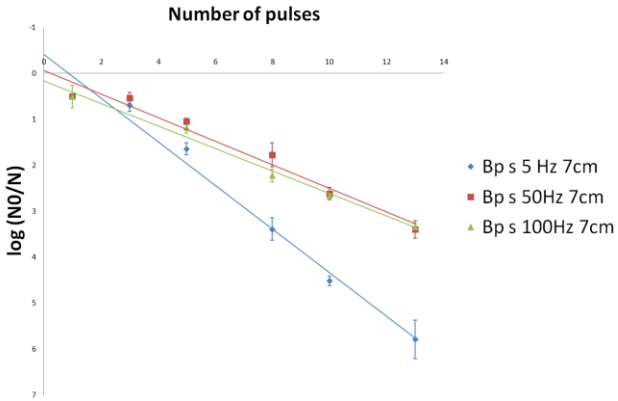

Fig. 1. *Influence of the pulsed repetition frequency on the treatment efficiency on Bacillus pumilus spores.* Effect of different number of pulses when samples were placed at 7centimeters from the extraction window and for different frequency (Hz): 5Hz (diamond), 50Hz (square) and 100Hz (triangle) on the inactivation rate.

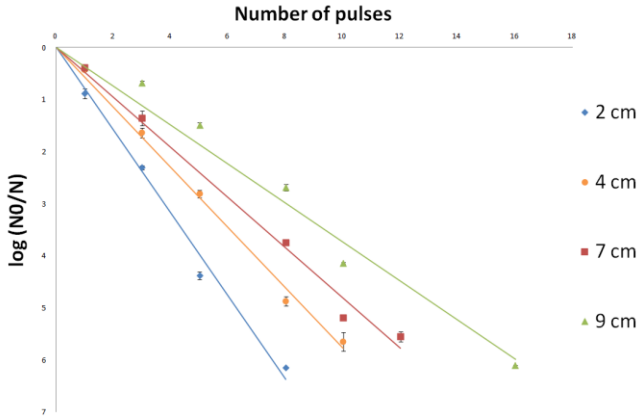

Fig. 2. *Influence of the distance from the extraction window on the treatment efficiency on Bacillus pumilus spores.* Effect of different number of pulses when samples were placed at different distances from the extraction window: 2 centimeters (diamond), 4 centimeters (round), 7 centimeters (square) and 9 centimeters (triangle) on the inactivation rate.

C. *Evolution of the resistance after several treatments at sub-lethal dose*

The efficiency of the technology was validated. However, it is important to know if any resistance can appear in the case of multi-exposure at sub-lethal dose. Fig.3 presents the logarithmic reduction obtained for the Wild type (WT) in comparison with the reduction of bacteria which had been irradiated at sub-lethal dose before (n+x). The letter x means the number of time that the solution had been exposed at sub-lethal dose before the treatment apply. The WT and the n+x had been placed under the electron beam at the same moment to ensure the same energy deposition between the reference and the assay. The results showed no difference of efficiency after 10 times of treatment. Pulsed electron beams do not induce resistance on *Bacillus pumilus* spores.

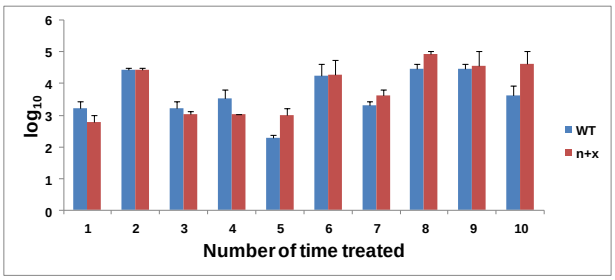

Fig. 3. *Treatment at sub-lethal dose not induced resistance.* Comparaison of the inactivation rate after 10 pulses at 7centimeters and a repetition frequency of 100Hz between bacteria which never see any irradiation treatment (WT) and bacteria which was treated x time(s) at sublethal dose (n+x).

D. *Cell wall observation by electronic microscopy*

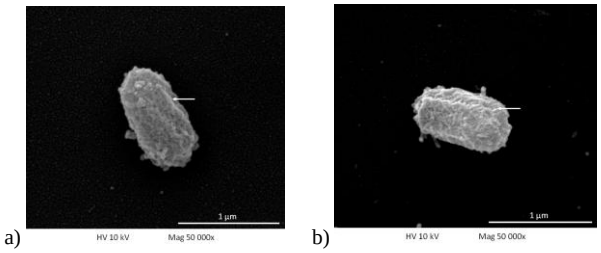

Fig. 4. *Observation of Bacillus pumilus spores wall by SEM.* The comparison of the non-treated spore (a) with the spore irradiated (b) did not reveal difference. Ridges (indicated with white arrows) were present for the two conditions.

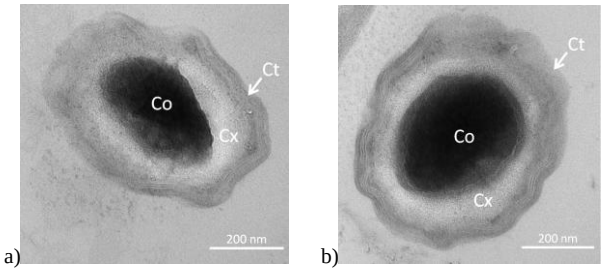

Fig. 5. *Observation of Bacillus pumilus spores wall by TEM.* The comparison of the non-treated spore (a) with the spore irradiated (b) did not reveal difference. The coat (Ct), the cortex (Cx) and the core (Co) are observable. The different layer of the coat are present in all case.

Spores were observed by SEM (Fig. 4) and TEM (Fig. 5). Ridges can be observed at the surface of non-treated and treated bacteria and none difference was observed.. To validate these observations, we performed TEM experiments. Indeed, the structure of the coat (Ct), the cortex (Cx) or the core (Co) can be observed and perfectly distinguished. The protein multilayers of the coat, the cortex and the core were present and do not seem altered after irradiation.

IV. DISCUSSION

Pulsed electron beam, is an efficient irradiation technology to kill bacteria even under spore form. *Bacillus pumilus* appears to be the most resistant bacteria, as previously described by U.S. Pharmacopeia. Moreover, experiments do not reveal an evolution of the resistance after 10 treatments under lethal dose. However, SEM and TEM, do not reveal any changes in the cell wall and organization after irradiation. These results are in contradiction with S.E. Fiester which shown damage on the spore coat by MEB [6]. The hypothesis is that, our technology uses high dose rate and could interact with molecules and cell wall differently. Consequently, to complete the cell wall analysis, Atomic Force Microscopy (AFM) on liquid medium will be used to measure, in living

condition and at the nanoscale, the structural organization and the mechanical properties of the cell wall after irradiation [11].

In other way, some scientists supposed that electron beam induced double strand break on DNA [5] [12] [13]. To validate this hypothesis on our technology, Pulsed Field Gel Electrophoresis (PFGE) will be used.

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