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Factors involved in *Bacillus* spore's resistance to cold atmospheric pressure plasma

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ABSTRACT

In this study factors involved in spore resistance to cold atmospheric pressure plasma (CAPP) were investigated. Therefore, wild-type *Bacillus subtilis* spores and isogenic mutant strains, PS578 lacking the genes encoding the spore's two major small acid soluble proteins (SASPs), FB122 being unable to synthesize dipicolinic acid (DPA) during sporulation and PS3328 lacking the outer spore coat, were CAPP treated using different process gasses (air, N_2, O_2, CO_2). Obtained inactivation depended on the process gas; highest inactivation efficiency was obtained with N_2-plasma. The results indicated that SASPs contribute in general to spores' CAPP resistance. DPA and outer spore coat were also important in the protection against UV photons, however, the protective effect was not so pronounced as for SASPs. Furthermore, they contributed in resistance against generated ozone. *Bacillus atrophaeus* spores, the surrogate for chemical and irradiation sterilization, showed over all a lower resistance to all tested CAPPs.

Industrial relevance: The application of cold atmospheric pressure plasma (CAPP) is an emerging low temperature technology for the inactivation of bacterial spores on different surfaces, such as food products as well as food contact surfaces and packing material. The results presented in this study help to understand the inactivation mechanisms and also the factors involved in the high resistance of bacterial spores to CAPP. This knowledge could be useful to optimize the plasma process for the decontamination of various surfaces in the food industry.

1. Introduction

The application of plasma at industrial scale to generate ozone and ultraviolet (UV) light has been used for decades in the food industry (Knorr et al., 2011). In 1857 Werner von Siemens designed a dielectric barrier discharge (DBD) plasma system to generate ozone, the so-called ozone tube was used to sanitize tap water (Siemens, 1857). Recently, cold atmospheric pressure plasma (CAPP) has gained importance as an emerging low temperature process for the inactivation of microorganism on food products and packing material as well as other food contact surfaces (Misra, Segat, & Cullen, 2015; Pankaj et al., 2014), especially for the decontamination of dry plant products like herbs and spices (Hertwig, Reineke, Ehlbeck, Erdoğdu et al., 2015), wheat grains (Butscher et al., 2015) and almonds (Deng et al., 2007). CAPP can be generated using different configurations, like DBD or plasma jet systems (Surowsky, Schlüter, & Knorr, 2014). In general, CAPP is a partially ionized gas, which contains different components; including neutral particles such as atoms, molecules and charged particles such as ions and electrons; furthermore, radicals, UV photons and also irradiated heat. Whereby, the different generated reactive components and their synergetic combinations are responsible for the antimicrobial effect of the CAPP treatment. Furthermore, the composition of the generated plasma depends on the used plasma source, process gas, type of application (direct or indirect) and operation conditions, e.g. energy input (Ehlbeck et al., 2011; Weltmann et al., 2008).

Bacterial spores belonging to class *Bacilli* or *Clostridia* are extremely resistant towards environmental stress conditions, including wet and dry heat, irradiation, UV, high pressure and chemicals (Setlow, 2014). Consequently, bacterial spores are adapted to survive on surfaces like dry food products or food production lines, thus being a major vector causing food spoilage, foodborne diseases and other human illnesses (Logan, 2012; Mallozzi, Viswanathan, & Vedantam, 2010). Various studies have already shown the potential of different CAPP applications to inactivate *Bacillus* spores on different surfaces (Butscher et al., 2015; Hertwig, Reineke, Ehlbeck, Knorr, & Schlüter, 2015; Hertwig, Steins et al., 2015; Kim, Lee, & Min, 2014; Reineke, Langer, Hertwig, Ehlbeck, & Schlüter, 2015). However, the mechanisms responsible for *Bacillus* spores' inactivation by CAPP are not fully understood. It is

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known that mechanisms, like etching, photodesorption and the action of UV photons are involved in the inactivation process (Moisan et al., 2002). Whereby, the UV photons can have a significant impact on the inactivation process due to DNA damage (Hertwig, Steins et al., 2015; Reineke et al., 2015). Wang, Doona, Setlow, and Li (2016) suggested that cold atmospheric plasma treatment damages spores' inner membrane and also key germination proteins, causing spore inactivation.

As previously mentioned *Bacillus* spores are extremely resistant towards various environmental stresses, whereby several factors, such as their multilayer morphology, are involved in their resistance. The spore coat, which contains a large amount of total spore protein, is a permeability barrier, thus restricting the access of large molecule like enzymes into the spore (Driks, 1999; Setlow, 2014). Furthermore, the coat protects the spore also against biocidal chemicals, probably due to detoxification (Setlow, 2006). The inner spore membrane also acts as a permeability barrier, limiting the access of toxic chemicals into the spore core. The core of the spore has a low water content, which is important for wet heat resistance. It also contains a spore unique acid, the dipicolinic acid (DPA), which protects spores' DNA against some damaging agents (Setlow, 2014). The DNA is saturated with α/β-type small acid soluble proteins (SASP), which protect the DNA against different kinds of damage, caused by various chemicals, wet and dry heat, UV photons and γ-irradiation (Fairheadt, Setlow, & Setlow, 1993; Setlow, 2014; Setlow & Setlow, 1993). Further on, the *Bacillus* spore is able to repair certain DNA damage during spore outgrowth (Setlow, 2014). Reineke et al. (2015) have already shown the contribution of SASPs and DPA to Bacillus spore's UV resistance, whereby the SASPs seem to play a more important role compared to the DPA.

In this study the inactivation behavior of *B. subtilis* spores to CAPP treatment was investigated. Further on, isogenic mutant strains were used to determine the spores's properties involved in the resistance to CAPP treatment, focusing on spores' outer coat, DPA and SASP. To distinguish between the different generated reactive species of the CAPP, regarding the inactivation process and resistant properties, different process gasses, such as dry air, N_2, O_2 and CO_2 were used to control the generation of these species during the CAPP treatment. The obtained inactivation data were compared with *Bacillus atrophaeus* spore inactivation.

2. Material and methods

2.1. Bacillus strains and spore preparation

In this study spores of *B. atrophaeus* strain WIS 396/3 (WIS Bundeswehr Research Institute for Protective Technologies and CBRN Protection), *B. subtilis* strain PS832 (wild-type) and three isogenic derivates of this *B. subtilis* strain, namely FB122 (Douki, Setlow, & Setlow, 2005), PS578 (Genest, Setlow, Melly, & Setlow, 2002) and PS3328 (Paidhungat, Ragkousi, & Setlow, 2001) were used. The spores of *B. atrophaeus* strain WIS 396/3 are used as surrogates for *B. anthracis* spores during chemical, UV- and γ-irradiation sterilization (Reineke et al., 2015). The strain FB122 (*sleB spoVF*) lacks the gene encoding the cortex lytic enzyme SleB and is not able to synthesize DPA during sporulation. The strain PS578 (α^- β^-) lacks the genes encoding the spore's two major α/β-type SASPs, thus the α/β-type SASP level in the PS578 spores is only ~25% of that in the wild-types spores PS832. The strains PS3328 (*cotE*) lack the CotE protein, which is essential for the spore's coat morphology (Henriques & Moran, 2007), especially for the structure of the outer spore coat (Driks, 1999). All spore strains were sporulated using a method previously published (Nicholson & Setlow, 1990). The sporulation was induced on 2 × SG medium agar plates at 37 °C without antibiotics. However, for the strain PS3328 tetracycline (Sigma Aldrich) was added at a concentration of 20 $\mu g * L^{-1}$. The obtained spore suspensions were washed and cleaned with cold distilled water by repeated centrifugation (three-fold at 5000*g*) and intermittently treated by ultrasonication for 1 min. The cleaned spore suspensions contained ≥ 95% phase bright spores and nearly no agglomerates. The spore suspensions were stored in the dark at 4 °C, until needed.

2.2. Sample preparation

In this study glass beads were used as a spherical model to investigate the inactivation of the different spore strains (Hertwig et al., 2015). The glass beads had a diameter of 5 mm. The samples were inoculated with a spore density of about $5 * 10^5$ to $6 * 10^6$ spore cm^{-2}. Therefore, 82 sterile glass beads were placed into a sterile beaker and 175 µL stock spore suspension was added. The beaker was placed on an automatic stirrer and shaken for 4 min at 400 rpm to obtain a homogenous coating of the microorganisms on the samples surface. The inoculated samples were placed in a biosafety cabinet for drying at room temperature for 30 min.

2.3. Plasma source and plasma treatment

For the CAPP treatment a diffuse coplanar surface barrier discharge 400 (DCSBD) plasma plate (CEPLANT, R & D Centre for Low-Cost Plasma and Nanotechnology Surface Modifications, Masaryk University, Brno, Czech Republic) was used, which is shown in Fig. 1 and described in detail by Černák et al. (2011). The plasma generated with the DCSBD plate consists of a high number of various microdischarges and creates an approximately 0.3 mm thick and homogeneous plasma layer. The plasma was generated by applying a sinusoidal high voltage (20 kV peak to peak and a frequency of 15 kHz) on the upper side of an Al_2O_3 ceramic and has no contact with the electrodes on the bottom side of the ceramic. A dielectric insulating oil circulation system was used for the electric insolation of the electrodes. The circulating oil was tempered using a plate heat exchanger and worked as a cooling system for the generated plasma. The application area of the plasma was 200 mm × 80 mm. For the experiments two different treatment chambers were used. The first reactor (Fig. 2A) consisted of one DCSBD plate and a glass cover with connections for the

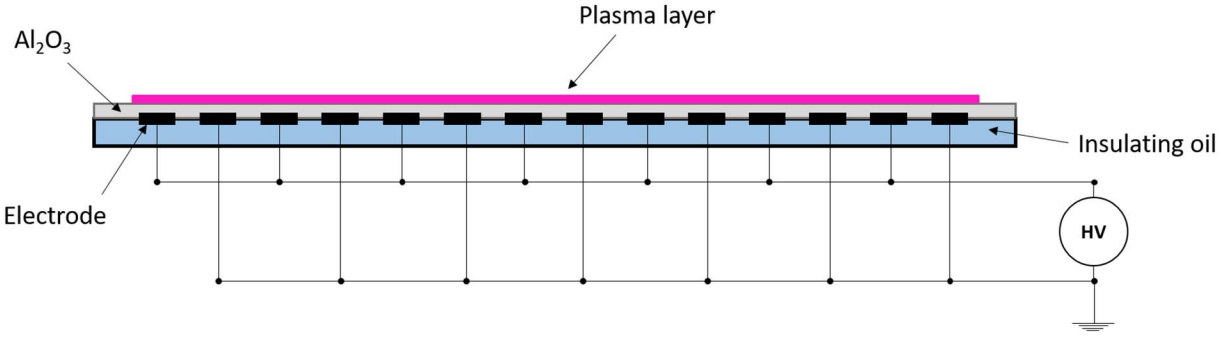

Fig. 1. Scheme of the DCSBD plate (Hertwig et al., 2017).