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Inactivation of Microorganisms in Tyvek® Packaging by Microwave Plasma Processed Air

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Abstract

Plasma is well-known for its antimicrobial capacity and moreover, it is successfully used in manifold industrial fields such as packaging industry. Microwave plasma processed gas with shares of reactive nitrogen species (RNS) was investigated for its decontamination efficiency against vegetative bacteria, conidia and bacterial endospores packed in Tyvek®. For all tested microorganisms, increased inactivation was found at prolonged treatment times. Furthermore, a treatment with moistened gas resulted in shorter treatment times and a dependency of humidity was observed. The inactivation rates increased up to 6 $\log_{10}$ steps. The microwave plasma processed gas showed very high microbial effects to vegetative bacteria, spores of *Bacillus atrophaeus* and *Aspergillus brasiliensis* in treatment times comparable to currently common methods like EO, FORM and hydrogen peroxide. Moreover, this new methode is charaterized by advantages like no thermal influences, no toxicity for human and environment and low costs.

Keywords: bacterial spores; microwave discharges; nitrogen; plasma treatment; sterilization.

1. Introduction

Plasma is used for the treatment and modification of surfaces at a variety of industrial applications, like etching, coating, cleaning and decontamination. Sterilization and decontamination of surfaces are a concern in many technological fields such as medical and food industries as well as packaging.[1-11] The last one is currently under active research because plasma allows a fast and safe treatment of packaging materials without influence to its properties. Furthermore, physical plasma has the potential of depyrogenation and inactivation of prions.[12]

Numerous of different atmospheric pressure plasma sources are available for application.[13] All of them are united by the composition of the plasma of molecules, radicals, ions, atoms, electrons and radiation. Gases such as oxygen or hydrogen peroxide in the plasma state have shown antimicrobial activity.[14 – 16] Furthermore, plasma scientists found manifold reactive species with antimicrobial capacities generated by atmospheric discharges.[17,18]

A lot of packaging materials are thermo- and hydro-sensitive. Materials such as polyethylene terephthalate (PET) and polyethylene (PE) are very important. Importance is increasing not only in food and beverage but also in pharmaceutical and medical industries. Low-temperature gas plasmas may be possible alternatives to other low-temperature decontamination procedures taking their drawbacks and limitations into account. Commonly used methods for thermo labile devices are ethylene oxide (EO), formaldehyde (FORM), gamma irradiation or hydrogen peroxide (H_2O_2). The disadvantages of high toxic EO are residues absorbed on the plastics and long storage times for venting. Another toxic and carcinogenic chemical is formaldehyde. Its usage requires particular handling, too. Radiation is costly and may modify materials.[19] Hydrogen peroxide and peracetic acid are the main utilized procedures for the decontamination of thermal-sensitive materials.[20] These harmful chemicals require again a particular handling and the removal of chemical residues from packaging.

However, plasma overcomes many of such limitations because of its compatibility with heat-sensitive materials, short treatment times, simple and safe user interfaces as well as no toxicity after processing. Many investigations for atmospheric pressure plasmas and their microbicidal effects have been done during the last decade.[13, 21-26] In the majority of these studies devices have been directly exposed to plasma, although customary wrapped medical instruments and other devices are sterilized.

With regard to practical use in medical facilities, decontamination of various kinds of medical instruments wrapped by sterilization bags is important. Therefore the development of plasma decontamination techniques to inactivate microorganisms inside specific plastic packaging, such as Tyvek® packaging, is essential.[27]

An advantage of plasmas gaseous form is the possibility to penetrate even in inhomogeneous surfaces and cavities down to the micrometer scale, at which traditional fluid or chemicals mostly fail. Plasma offers the possibility of varying parameters like process gas, pressure or applied power density. Air seems to be very efficient for microbiological inactivation by the reason air plasmas are excellent sources of reactive oxygen-based and nitrogen-based species, such as O, O_2*, O_3, OH•, NO• and NO_2•.[28] Therefore, plasma could offer a simple, fast and effective disinfection of medical devices, surfaces and other equipment in an economical way.[29]

In our study we investigated the inactivation of different microorganisms caused by microwave plasma treatment. To analyze the antimicrobial efficiency of the plasma gas, it is necessary to investigate a wide range of microorganisms for optimization and validation of a newly developed decontamination method. Therefore, endospores of *Bacillus atrophaeus* (ATCC 9372) and conidiospores of *Aspergillus brasiliensis* (ATCC 16404) as well as the vegetative bacteria *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* K-12 (NCTC 10538) were used. Furthermore, the amount of humidity may play a role for improving microbicidal effects, hence, this hypothesis was also investigated within this work.

2. Experimental Part

2.1 Investigated strains

Bacillus atrophaeus Nakamura 1989 (ATCC 9372) in its sporolated form was used and the vacuum dried culture achieved from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). It was cultivated on sporulation agar for two weeks at 37 °C and after harvest stored in a refrigerator until use to prevent spore germination. *Aspergillus brasiliensis* (ATCC 16404) was also achieved form the DSMZ and kindly prepared and eluted by the HygCen GmbH (Centrum für Hygiene und medizinische Produktsicherheit, Schwerin, Germany). *Escherichia coli* K-12 (NCTC 10538) and *Staphylococcus aureus* (ATCC 6538) have been kindly provided by Institute of Hygiene and Environmental Medicine, Ernst Moritz Arndt University Greifswald, Germany.

2.2 Contamination of specimens

In preparation for the plasma treatment specimens were contaminated with microorganisms. As specimens glass slides with a size of 32x8x2 mm were used. All microorganisms, vegetative germs or spores were diluted in distilled water in concentrations of 10^8 cfu ml^{-1}. Glass slides were contaminated under laminar flow by pipetting. Next, the dried specimens were weld in flat reels, which are made by Tyvek® rolls (ASP c/o Ethicon GmbH, 22844 Norderstedt, Germany). Such prepared slides were set into glass bottles before plasma treatment took place.

2.3 Treatment by microwave plasma processed gas

The plasma treatment of the contaminated specimens was done with plasma processed gas air generated by microwave plasma source. The used microwave plasma setup is shown in **Figure 1**. The microwave has a frequency of 2.45 GHz and the consumed power is in the range of 1.1 kW. Accordingly, the gas temperature is about 5000 K at a gas flux of 16 slm compressed air (dew point at + 3 °C). The distance between the torch and the contaminated glass bottles is about 25 cm connected via a metal tube. The gas temperature at the end of the metal tube is 120 °C. In contact with the bottles the gas temperature is immediately cooled down to room temperature. Since the main decontamination process is induced by plasma chemistry the discharge is ignited for 7 s, the reactive gas is introduced into the glass bottle. Only for plasma treatment, bottles were opened, apart from that they stayed closed. The plasma gas could interact with microorganisms for 10 and 30 seconds as well as 1, 2, 5, 10, 15, 30, 45 and 60 minutes through Tyvek®.

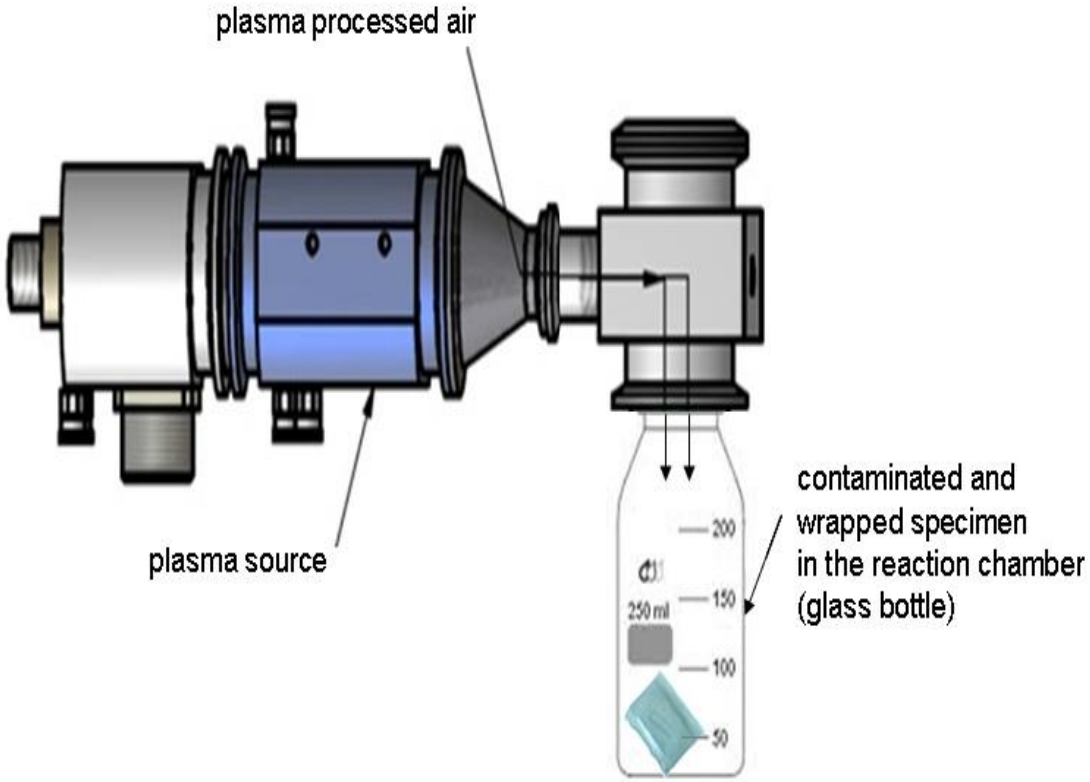

Figure 1. The microwave setup for decontamination of Tyvek® wrapped specimens.[49, 50]

2.4 Recovery of microbial contamination

The recovery of potential residues followed by shaking the bottles with 10 ml tryptic soy broth (CASO-Bouillon, Merck KGaA, Darmstadt, Germany) for 15 minutes. In the case of *B. atrophaeus* spores treated bottles were heated for 10 minutes at 80 °C after shaking to destroy any vegetative bacteria. The remaining concentration of residues was detected by the surface-spread-plate count method using tryptic soy agar plates (CASO-Agar, Merck KGaA, Darmstadt, Germany). The detection limit of this procedure was 10 cfu ml^{-1}. Inactivation kinetics of microorganisms are depicted in semi-logarithmic plots. If the number of microorganisms fell below the detection limit, i. e. no viable microorganisms have been found, these values in the graphs are set at the detection limit.

2.5 Temperature measurements

The used plasma set-up PLexc® is a microwave plasma source which provides plasma processed air with temperatures up to 5000 °C. To measure the temperature the specimens are placed inside the beaker and pictures were taken by a thermal imaging camera (FLUKE Ti20 Thermal Imager) during plasma treatment. The temperature range of this camera is between -10 °C and 350 °C. 256 colour shades can be detected and shown.

2.6 Chemical composition of used plasma gas

Mass spectrometry was used to analyse the composition of the microwave discharge generated plasma gas. The used mass spectrometer was OmniStar™ GSD3001O1 from Pfeiffer Vacuum (35614 Asslar, Deutschland). The spectrometer consists of a heatable analysing chamber with a quadrupole as ion-selective element und a Faradaycup as detector. The measurements were done with a switched-on filament. For analysis under atmospheric pressure the plasma gas were run through a heatable stainless steal capillary (inner diameter 1/16" with a length of 1 m) into the analysing chamber. Thereby, a gas flow of 1 to 2 sccm is generated. The detection range of the mass spectrometer was 1 to 100 amu. The measurements and evaluations were done by software QuadStar™ from Pfeiffer Vacuum. Calibration of mass spectrometer for mass scale by reference gases and detector flow took place before measurements were started. Water deposits were avoided by heating the chamber and the capillary over days. Background spectra were taken before and after the plasma process to ensure thatchanges in concentrations are caused by plasma.

3. Results and Discussion

3.1 Temperature measurements

Figure 2 shows the results for temperature measurements. In this case the treatment of a glass beaker with a volume of 250 ml comparable to the glass bottle used in biological experiments was used. The scale of temperature starts at 20.8 °C (black) and ends at 38.5 °C (red). It can be seen clearly, that the beaker is heated from 23.8 °C up to 25 °C maximum at the nearest area to the plasma gas entrance. The measured temperatures are compatible to the thermo-labile materials such as Tyvek® and with all investigated bacteria, conidia and endospores. Temperature effects were excluded, thus, the observed antimicrobial effects are referred to plasma gas components.

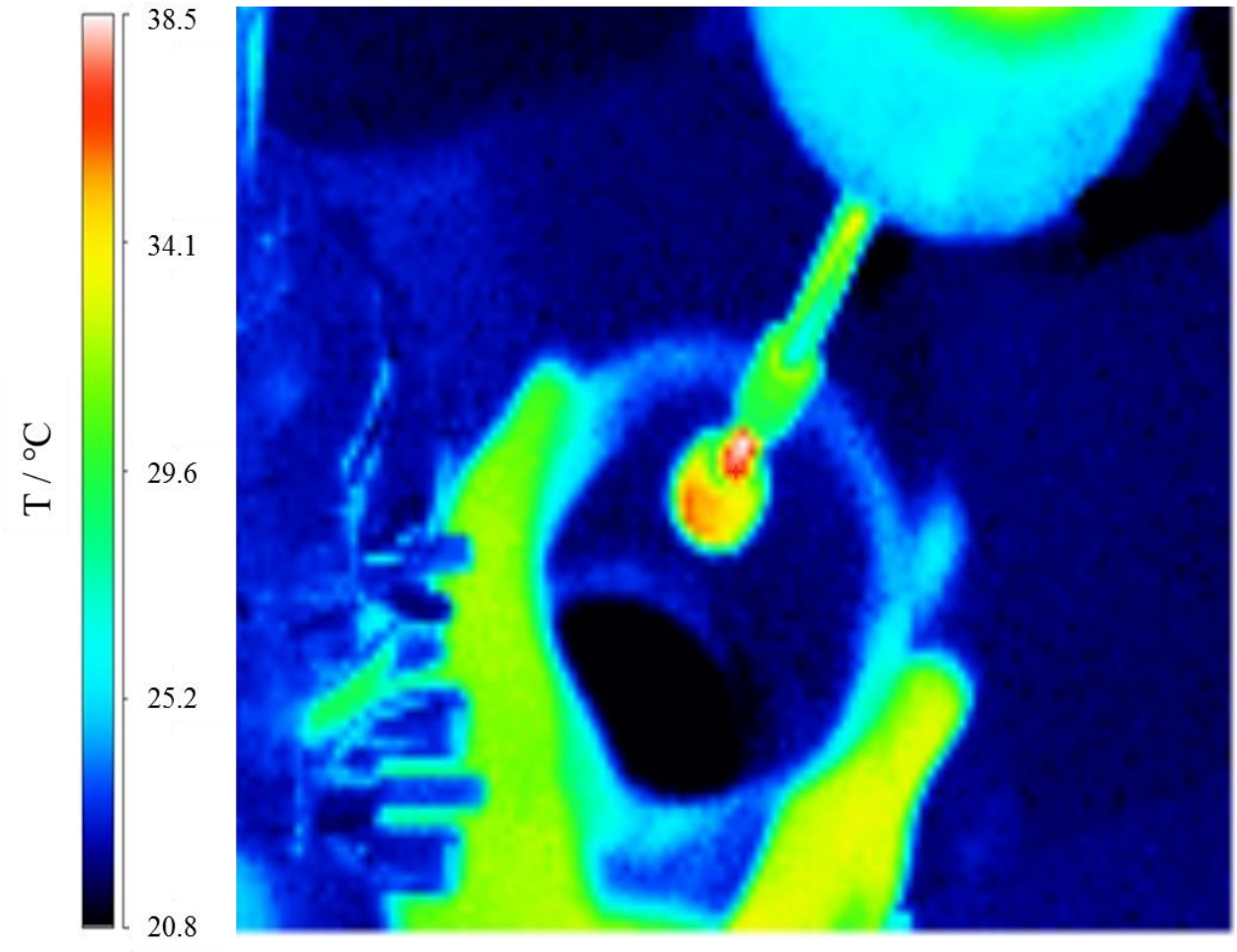

Figure 2. Temperature measurements of a beaker during plasma treatment. Green parts are the operators hand and parts of plasma source.

3.2 Chemical composition of used plasma gas

By mass spectrometry the composition of the microwave plasma processed gas was analyzed. After the plasma process 3 % of the working gas (compressed air at 23.8 °C room temperature) remains changed according to the regarding time scale. The generated products were stable for at least 60 minutes. The gas composition is shown in **Figure 3**. The plasma processed 2.6 % of compressed air to 6 different molecules and radicals. The highest shares belong to nitrogen monoxide and dioxide (NO•, NO_2•). Other parts are CO_2 (0.07 %), H_2O (0.03 %), HNO_3 (0.04 %) and HNO_2

(0.09 %). Antimicrobial effects can be estimated by the radicals and the acids. Furthermore, instable and metastable intermediates are surely probable, but currently not detectable.

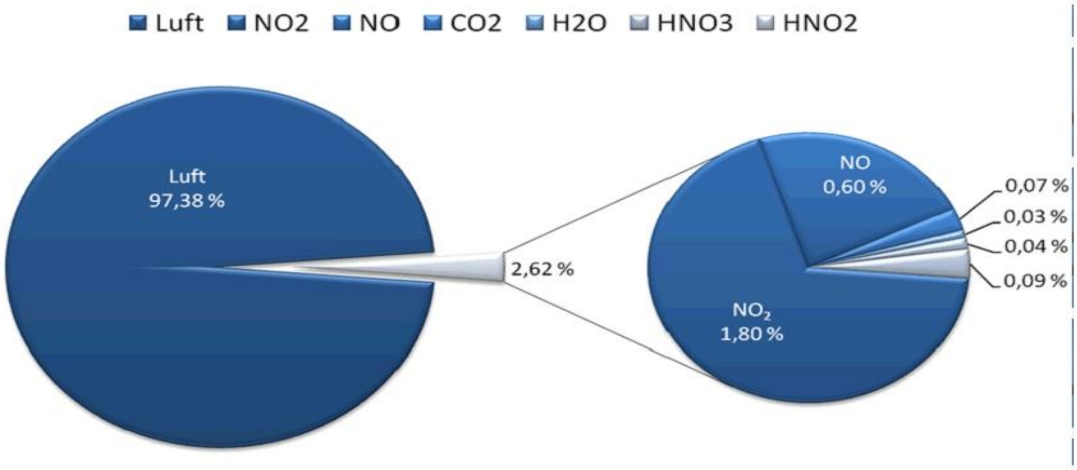

Figure 3. Chemical composition of microwave plasma processed air.

3.3 Inactivation of vegetative bacteria

To investigate the antimicrobial efficacy of plasma gas through Tyvek® primarily *E. coli* and *S. aureus* were chosen as vegetative germs. In **Figure 4** the survival curves for both bacteria in time depended plasma gas treatment are shown. It can be observed that the surviving curve of *E. coli* decreases over time and therefore, the antimicrobial capacity of plasma gas is increased with longer treatment times. This effect is very evident between the initial time point and 10 minutes. Afterwards, the decrease in survivors is prolonged until the detection limit of 10 cfu ml^{-1} after 60 minutes treatment time. The diminish in the last 50 minutes is very constant and unexpected. The treatment of *S. aureus* was done under the same conditions like *E. coli*. Unlike the last, *S. aureus* requires shorter treatment times. The exposure of bacteria to the plasma gas for 10 minutes led to an inactivation of almost 4 $\log_{10}$ steps. The strong decrease in the first 10 minutes is then weakened until no residues could be detected until the limit after 30 minutes. This indicates that the mortality of vegetative bacteria increases by elevating the treatment time up to 5 $\log_{10}$ steps. Moreover, the level of inactivation was not proportional to the time treatment, which indicates a multi-phased progression of the kinetic. This phenomenon is also described by others.[30] This may be in correlation with the observation that plasma has a limited penetration depth and therefore stacked bacteria or surface layers gradually slow down the inactivation process. Moreover the Tyvek® can be seen as an additional diffusion barrier for the gas.

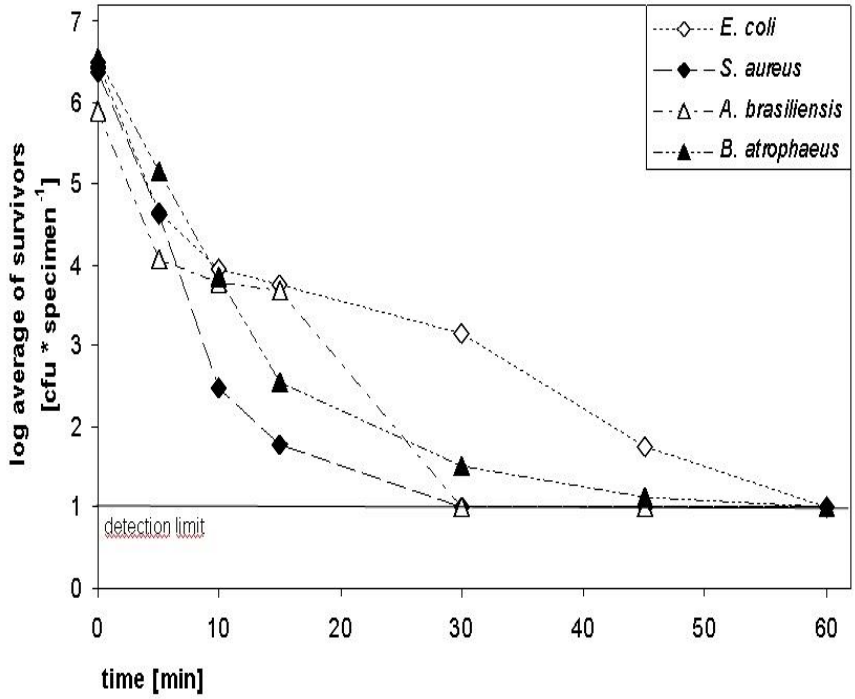

Figure 4. Effect of different incubation times on inactivation of *Escherichia coli* (◇), *Staphylococcus aureus* (◆), conidia of *Aspergillus brasiliensis* (△) and endospores of *Bacillus atrophaeus* (▲) with plasma processed air. Specimens are wrapped in Tyvek®. References without plasma treatment are set at time point t_0. Values are the average of triplicate.

3.4 Inactivation of spores

To investigate whether the cocktail of reactive species generated by plasma is able to inactivate a wide range of microorganisms, *Aspergillus brasiliensis* conidiospores and *Bacillus atrophaeus* endospores were used. Conidiospores (conidia) are built by fungi. They are generated through the cellular process of mitosis and can develop into new organisms if conditions are favorable, and serve in biological dispersal. Endospores are built by some bacteria. They are dormant, tough, and temporarily non-reproductive structures. It is a stripped-down, dormant form to which the bacterium

can reduce itself. Endospore formation is usually triggered by a lack of nutrients. Endospores enable bacteria to lie dormant for extended periods. When the environment becomes more favorable, they can reactivate themselves to the vegetative state. Endospores are resistant to ultraviolet radiation, desiccation, high temperature, extreme freezing and chemical disinfectants. The results for these experiments are shown in **Figure 4**. In a time series up to 60 minutes treatment time the gas was capable to inactivate the conidiospores until the detection limit of 10 cfu ml^{-1} was reached. After 5 minutes of treatment the viability of the conidiospores was reduced about almost 2 log_{10} steps. Furthermore, the reduction increases after a total treatment time of 15 minutes and more to a maximum of 4.8 log_{10} steps after the detection limit was reached. Between the treatment time of 5 to 15 minutes an interruption of microbicidal effects was observed, resulting in a plateau. This three-step inactivation curve was only detected for the investigated fungal spores. The last curve which is shown in **Figure 4** is the one of Tyvek®-packed endospores of *B. atrophaeus*. The longer the treatment time the endospores are contacted with the plasma gas, the higher the inactivation value. As *Bacillus* spores are highly resistant to many disinfectants a longer treatment time for a complete inactivation until the detection limit of 10 cfu ml^{-1} was expected. In relation to that, the number of survivors first fell under the detection limit after 60 minutes. During the first 15 minutes a nearly linear reduction of 4 log_{10} steps were observed, but in the next 45 minutes the curve decreased only slowly. This two-step curve indicates a second-step mechanism of inactivation, thereby, in the first phase isolated spores or the first layer of stacked spores is influenced and in the second phase, which had the slower kinetic, the conversion of NO• to NO_2• took place.[16, 31] Furthermore, the barrier presented by Tyvek® must be overcome by the microbicidal gas components.

3.5 Influence of humidity

Investigation of the antimicrobial efficiency of plasma gas through Tyvek® in dependence of humidity was done next. Wrapped specimens contaminated with *E. coli*, conidia of *A. brasiliensis* or endospores of *B. atrophaeus* were used. In **Figure 5** the survival curves for bacteria and spores in time depended plasma gas treatment are shown. Longer treatment times led to higher inactivation rates of *E. coli*. This effect is obviously between the initial time point and 2 minutes. Afterwards, the decrease in survivors reached the detection limit of 10 cfu ml^{-1}. In the first two minutes a nearly linear reduction of *E. coli* was detected. During the initial 10 seconds a 1.5 log_{10} step decrease was achieved, followed by a further log_{10} step after 30 seconds and resulted in 3.4 log_{10} steps after 60 seconds. The detection limit was reached after 2 minutes and a prolonged treatment time approved this result.

The further investigated spores, conidiospores of *A. brasiliensis* and endospores of *B. atrophaeus*, showed a clear decrease of viability, too. Demonstrated in **Figure 5** the treatment with moistened plasma gas (dew point at + 9.4 °C) led to complete inactivation of the conidia after a treatment time of 60 seconds. The detection limit of 10 cfu ml^{-1} was reached. After 10 seconds of treatment time the viability of the contaminants was reduced about almost 1.5 log_{10} steps, furthermore, the reduction increases after a treatment time of 30 seconds and more to a maximum of 5.0 log_{10} steps. Within the first 30 seconds a very strong and nearly linear decrease was observed and in the next 30 seconds the last conidia of about half an order of magnitude were inactivated. This linear inactivation curve was also seen for the endospores of *B. atrophaeus*. However, the lower defence mechanisms of the *A. brasiliensis* conidiospores resulting in its inactivation twice as fast as the one of the endospores.

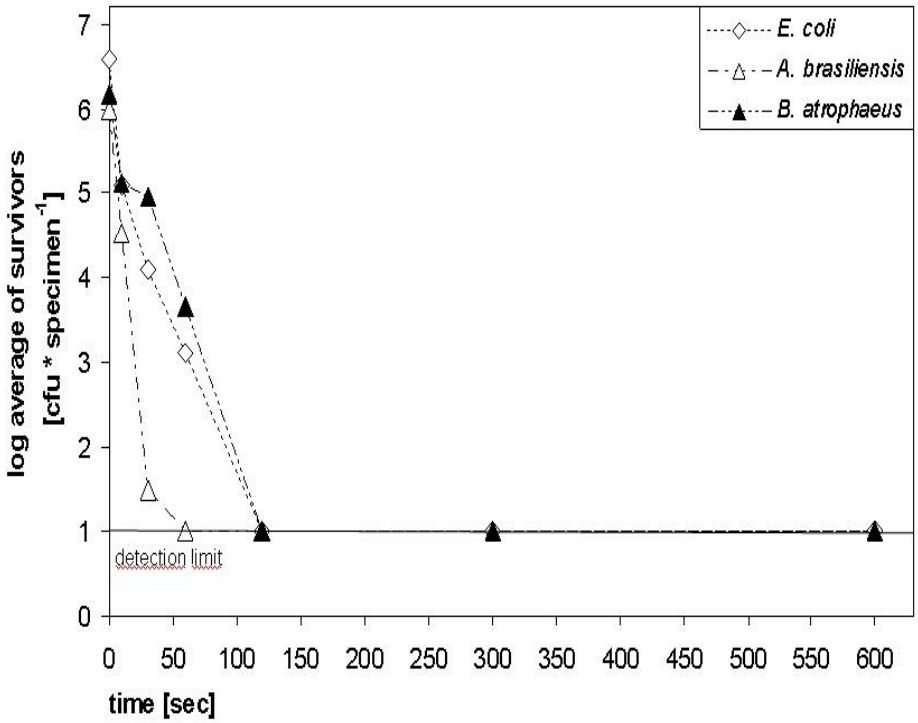

Figure 5. Effect of different incubation times on inactivation of *Escherichia coli* (◇), conidia of *Aspergillus brasiliensis* (△) and endospores of *Bacillus atrophaeus* (▲) with plasma processed air and humidity at a dew point of + 9.4 °C. Specimens are wrapped in Tyvek®. References without plasma treatment are set at time point t_0. Values are the average of triplicate.

Humidity itself fasten the microbicidal effects of plasma gas. However, the amount of humidity plays also a role. **Figure 6** shows that clearly. A lower humidity at a dew point of + 2.6 °C resulted in a decrease of *B. atrophaeus* endospores of 4.12 $\log_{10}$ steps after 5 minutes and a maximum of 4.2 $\log_{10}$ steps after 10 minutes. The detection limit was not reached. Contrary, a humidity at a dew point of + 6.9 °C led to an inactivation of 4.0 $\log_{10}$ steps within 90 seconds and the detection limit is reached after 120 seconds with a reduction of more than 6 $\log_{10}$ steps.

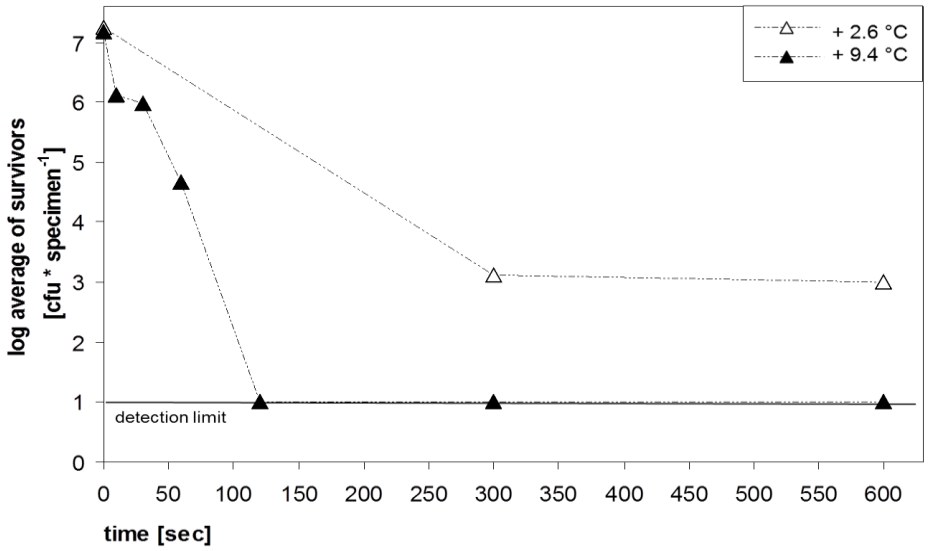

Figure 6. Effect of different incubation times on inactivation of endospores of *Bacillus atrophaeus* for humidity at a dew point of + 2.6 °C (△) and of + 9.4 °C (▲) with plasma processed air. Specimens are wrapped in Tyvek®. References without plasma treatment are set at time point t_0. Values are the average of triplicate.

The aim of this study was to investigate the microwave plasma inactivation efficiency by plasma gas against vegetative bacteria, conidia and bacterial endospores with a packaging out of Tyvek®. *B. atrophaeus* endospores were chosen due to their high resistance to disinfectants. Furthermore, *A. brasiliensis* conidiospores were taken to investigate a UV-resistant fungus and important microorganism in beverage industry. To examine the influence of the plasma gas to a wide range of bacteria simultaneously, *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) were selected.

The properties of gas plasmas can be modified and optimized by varying the main parameters such as pressure, power and process gas. Each alteration of one of these parameters changes the whole plasma chemistry and influences the electron density, concentration of charged or reactive particles and the amount of emitted UV radiation. Muranyi et al. (2007) had shown in previous studies that laboratory air is very effective for inactivation of microorganisms compared to other gases.[28] In this work, we have tried to improve the inactivation efficiency by taking air-generated RNOS of the gas phase after the plasma ignition.

The studies have shown that the inactivation of vegetative germs can be improved by increasing the treatment time. The highest inactivation for *E. coli* of 5.4 $\log_{10}$ steps was found at a treatment time of 60 minutes in plasma gas. For *S. aureus* the highest inactivation of 5.3 $\log_{10}$ steps was detected at a treatment time of 30 minutes. The necessary exposure time for *A. brasiliensis* conidiospores to reach the highest value of inactivation (4.8 $\log_{10}$ steps) was also 30 minutes. As shown by the inactivation kinetics, the effect was enhanced at prolonged exposure of microorganisms to plasma gas which indicate a time-dependent reaction. The faster inactivation of vegetative germs and conidiospores than of endospores was expected and detected, indicating the missing of potent defence mechanisms such as thick cell walls and membranes for the first ones. In support of this thesis, the complete inactivation of *B. atrophaeus* endospores was not attained before a 60-minute-treatment. Structure and chemical composition of endospores play a major role in their resistance. These endospores have a very different structure than vegetative cells or conidia. The complex structure of the spore-specific coat, cortex, inner spore membrane and the core itself with DPA (dipicolinic acid) and SASPs (small acid soluble proteins) elevate the resistance of bacterial spores against any inactivating agent compared to vegetative germs.[32 – 40]

Plasma has the potential to inactivate microorganisms by breaking or disrupting the cell wall without causing damage to the surround material.[41-43] Researchers postulated the rupture of the outer cell membrane after short plasma exposure, which causes leakage of the cytoplasm, where as longer plasma exposure triggers cell fragmentation of the spore. Both mechanisms result in cell death.[42, 43]

The observed survival kinetics suggest an inactivation mechanism by diffusion properties of the major gas components NO• and NO_2• for treatment without additional humidity. Minor compounds such as CO_2, H_2O, HNO_3 and HNO_2 may play a subsidiary role. The lower diffusion coefficient of NO• possibly leads to the small decrease seen in the first 5 minutes, afterwards the obtained plateau may be a result from the alteration of the metastabil NO• to the stabile NO_2• (1).

$$2\ NO\bullet + O_2 \leftrightarrows 2\ NO_2\bullet + 114.2\ kJ \qquad (1)$$

Finally nitrogen dioxide induces the second greater decrease and inactivation of the cells. We assume that the positive effect arises from the diffusion of nitrogen monoxide and nitrogen dioxide into and through the tested microorganisms. However, other radicals might be important, too, but were not detected by mass spectrometry.

Singh et al. (2009) showed that the inactivation efficiency was significantly affected by Tyvek® packaging for spores.[44] They assumed that it partially blocked the active species as well as reduced the UV emission intensity. From their optical emission spectroscopy measurement, it was confirmed that roughly 10 % of UV emission can transmit through the Tyvek®. In our case the UV emission was excluded. However, a diffusion barrier by Tyvek® packaging was created. The necessary treatment times to reach the detection limit are doubled comparing wrapped specimen with unwrapped. We could show complete inactivation reaching the detection limit of 10 cfu ml^{-1} after 15 minutes for vegetative bacteria as well as conidia and after 30 minutes for *B. atrophaeus* endospores.[45] Using Tyvek® as a diffusion barrier for plasma gas, the necessary treatment times increased to 30 minutes for *S. aureus* and *A. brasiliensis* conidiospores and to 60 minutes for the endospores.

Chemical reactions of air components with water molecules to possible products like nitrous acid, hydroxyl radicals and hydrogen peroxide were achieved by moisten the air with humidities at dew points of + 3 °C or + 6.9 °C. The literature pointed out an improved inactivation at 70 % humidity (dew point of + 15.3 °C).[46] Others could support this assumption.[47, 48] In our set-up moistening led to reduced treatment times humidity at dew point of ^+ 6.9 °C. A strong dependence between the amount of humidity and length of treatment time was detected. Using moistened plasma gas the required treatment times were reduced about 15 times less for *B. atrophaeus* endospores than in dry plasma gas. The observed survival kinetics suggest an inactivation mechanism by the major gas components NO• and NO_2• and more important by acidification (HNO_2, HNO_3) and possibly hydrogen peroxide. As H_2O_2 only exist in combination with water, we assume its existence in form of condensate. This condensate is maybe built as a thin film on investigated specimen. However, it was not measurable by mass spectrometry due to degradation processes or by commercial test kits, which detect unspecific OH-bounds.

A lot of chemical reactions may be possible inside the different phases created by dry and moistened plasma gas as it can be seen in **Table 1**. Main resulting products could be NO•, NO_2•, NO_3•, O_2•$^-$, O• and N_2O_3 for the gas phase; HO•, HNO_2 and HNO_3 for the gas- water-phase and H_2O_2, ONOOH, HNO_2 and HNO_3 for the water phase. All these components may be able to act as a disinfectant or sterilizing agent against microorganisms.

Table 1. Possible chemical reactions inside the processed gas.

gas phase	gas – water phase	water phase
$N_2 + O_2 \rightarrow 2\ NO^{\bullet}$	$H_2O \rightarrow H^+ + OH^-$	$O_2^{\bullet -} + e^- + 2\ H^+ \rightleftharpoons H_2O_2$
$2\ NO^{\bullet} + O_2 \rightleftharpoons 2\ NO_2^{\bullet}$	$H_2O \rightarrow H^+ + HO^{\bullet} + e^-$	$HO^{\bullet} + e^- + H^+ \rightleftharpoons H_2O$
$NO^{\bullet} + O_3 \rightarrow NO_2^{\bullet} + O_2$	$H_2O \rightarrow H^+ + HO^+ + 2\ e^-$	$HO^{\bullet} + O_3 \rightleftharpoons HOO^{\bullet} + O_2$
$NO^{\bullet} + NO_2^{\bullet} \rightleftharpoons N_2O_3$	$H_2O + O^{\bullet} \rightleftharpoons 2\ HO^{\bullet}$	$2\ HOO^{\bullet} \rightleftharpoons H_2O_2 + O_2$
$N_2O_3 + O_3 \rightleftharpoons N_2O_6 \rightleftharpoons 2\ NO_3^{\bullet}$	$4\ NO^{\bullet} + O_2 + 2\ H_2O \rightleftharpoons 4\ H^+ + 4\ NO_2^- / 4\ HNO_2$	$H_2O_2 + e^- + H^+ \rightleftharpoons HO^{\bullet} + H_2O$
$NO^{\bullet} + O_2^{\bullet -} \rightleftharpoons ONOO^-$		$HO^{\bullet} + NO_2^{\bullet} \rightleftharpoons ONOOH$
$ONOO^- + CO_2 \rightleftharpoons ONOOCO_2^-$	$4\ NO_2^{\bullet} + O_2 + H_2O \rightleftharpoons 4\ H^+ + 4\ NO_3^- / 4\ HNO_3$	$ONOOH \rightleftharpoons HNO_3 \rightleftharpoons NO_3^- + H^+$
$ONOOCO_2^- \rightleftharpoons NO_2^{\bullet} + CO_3^{\bullet -}$		$ONOOH \rightarrow 2\ NO_3^{\bullet} + HO^{\bullet}$
$2\ O_2 \rightarrow O^{\bullet} + O_3$		$NO_3^{\bullet} + NO_2^{\bullet} + H_2O \rightleftharpoons N_2O_5 + H_2O$
$O_2 + e^- \rightleftharpoons O_2^{\bullet -}$		$N_2O_5 + H_2O \rightleftharpoons 2\ NO_3^- + 2\ H^+$
$O_3 \rightarrow (O) + O_2$		$NO_2^{\bullet} + H_2O_2 \rightarrow [\ NO_2^- + H_2O_2^{\bullet +}] \rightarrow NO_2^- + O_2^{\bullet -} + 2\ H^+$
		$HNO_2 \rightarrow H_2O + N_2O_3$
		$N_2O_3 + HO^{\bullet} \rightleftharpoons 2\ NO_2^- + 2\ H^+$

4. Conclusion

The atmospheric pressure plasma set-up which we used within this publication allows a cold decontamination process without usage of toxic chemicals. An adequate treatment time common for current gas sterilization (FORM, peracetic acid, hydrogen peroxide and EO), up to 12 hours, is achieved and moreover strongly undercut due to optimized process conditions. The results demonstrate that a plasma process is capable of decontaminating thermo-labile packaging materials such as Tyvek® in accordance with the requirements defined by Food and Drug Administration (FDA), European Directorate for the Quality of Medicines and HealthCare protects (EDQM) and European Food Safety Authority (EFSA) and confirm a state of antimicrobial inactivation by means of challenge tests. Plasma decontamination was achieved using dry and moistened plasma gas. The treatment of *B. atrophaeus* endospores, conidia of *A. brasiliensis* and vegetative bacteria suggested that low-temperature decontamination was successful for a treatment time of 15 respectively 30 minutes. Moreover, reactive species have also microbicidal effects to *B. atrophaeus* endospores in a 15 times decreased treatment time (maximum 2 minutes) using humidity. The diffusion properties of the plasma gas implicit a possible gas transport into tiny gaps, long lumen and through diffusion barriers such as Tyvek®. The experimental results indicate the possibility of a plasma gas with no toxic residues, low costs and short treatment times to

decontaminate surfaces and lumen which can be found at medical devices, pharmaceutical, packaging and food industries.

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