The Microbicidal Effect of Low-Temperature Plasma Generated by Corona Discharge: Comparison of Various Microorganisms on an Agar Surface or in Aqueous Suspension

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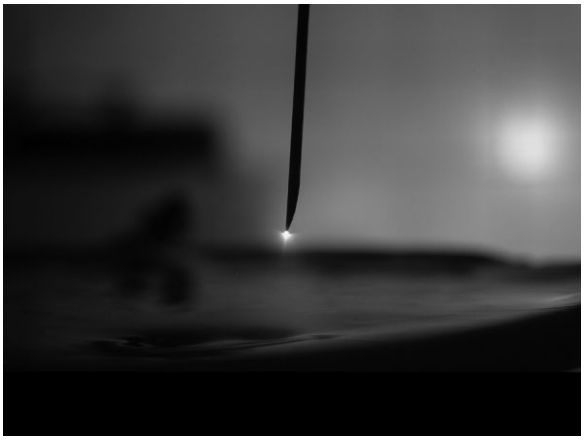
The sensitivity of various microbes to the low temperature plasma generated by negative corona discharge in the point-to-plane mode was tested. On the surface of agar semisolid cultivation media, the inhibition zones in microbial cultures were obtained and their areas were compared as a measure of microbicidal effect for nine microbial species including bacterial spores and a yeast species. This experimental arrangement resembles the testing of antibiotic sensitivity by the well-known disc diffusion technique. The effect of comparable magnitude was found for all vegetative forms, but *Candida albicans* and *Deinococcus radiodurans* appeared to be the most and *Geobacillus stearothermophilus* the least susceptible. The effect on spores was in order of magnitude lesser than on vegetative forms. Following the action of the same plasma on bacteria in aqueous suspensions, the complete sterilization of *Escherichia coli* was achieved within 120 s and *Staphylococcus epidermidis* within 4–5 min. On the other hand, the yeast *Candida albicans* in water appeared to be less susceptible requiring 30 min for its complete inactivation.



Introduction

The action of the non-thermal plasma (NTP) as one of the possible methods of decontamination or sterilization was studied intensively in recent years, the literature concerning this topic is very complex and diverse. NTP effects on biological samples depend on source of plasma and on composition of atmosphere or its pressure.[1] Concerning the mechanism of microbicidal action of various plasma types, its mediation by reactive particles (ions, radicals, and molecules generated in air mainly from oxygen and nitrogen) and, in lesser extent by the UV light is widely

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accepted. The state-of-the-art in NTP generation and use in microbiology and other biological and medical disciplines were many times thoroughly reviewed.[2–5]

Among various sources of NTP described and used in many previous works, Laroussi[6] used the radio frequency (RF) glow discharge. Akishev et al.[7] used two experimental arrangements: i) pulsed streamer-spark discharge in air-bubbled water was used for sterilization in liquids; ii) DC plasma jet with various gas mixtures was used for sterilization of cultivation media surfaces. This work demonstrated deep and fast inactivation by NTP at atmospheric pressure for resistant organisms including *Bacillus subtilis*, *Escherichia coli*, *Serratia marcescens, Mycobacterium flavescens, Candida lipolytica* (sic, probably *Yarrowia lipolytica*), and mixed bacterial biofilms composed mainly of sulfate-reducing bacteria. Stoffels et al.[8] developed the plasma needle (plasma jet), working with plasma generated in a stream of gas by 200–500 V RF. Many authors, e.g.,[9] further used this tool. Coulombe et al.[10] described some of its medical applications. The dielectric barrier discharge (DBD) seems to be a very promising tool for sterilization by NTP, its applications were reviewed by Fridman et al.[11] The later report of Cooper et al.[12] demonstrated the efficiency of DBD on *Deinococcus radiodurans*. Noyce and Hughes,[13,14] described some possibilities of sterilization by ions generated by DC current. The studies of microbicidal effect were focused mainly on bacteria, but applications on fungi and insects (e.g., ref.[15]) were also described. There are also commercial sterilization systems Sterrad (Johnson & Johnson) and Plazlyte (AbTox), employing radicals generated by RF from hydrogen peroxide and peracetic acid, respectively.

In the majority of the above-mentioned and other works, the alternating current mostly at RF or microwave frequencies was used to generate the plasma. We used the corona discharge burning at atmospheric pressure in air as one of the simplest and well-reproducible source of NTP. In contrast to other kinds of discharges, which usually require a construction of a complex apparatus, it can be generated in a simple device with negligible first expenses and operating costs. It is therefore suitable as a model for experimental research into the microbicidal properties of electric discharges. On the other side, its main disadvantage is the action in limited area only with non-uniform treatment. The properties of this type of discharge were also described by Sigmond et al.[16]

In our previous work,[17] we applied the direct high voltage to generate the plasma by the corona discharge in open air and used the inhibition zones on the surface of agar cultivation medium as a measure of microbicidal effect. This experimental arrangement resembles the disc diffusion technique or the E-test, widely used in medical microbiology for determination of antibiotic sensitivity of pathogens: The method is based on the fact that water-solutable antibiotic diffuses gradually through the agar from the test disc saturated with the tested antibiotic and laid on the surface of agar. That leads to the formation of antibiotic concentration radial gradient around the disc. Inoculated sensitive pathogens growth is inhibited starting from some minimal antibiotic concentration occurring at radius of inhibition zone. This zone is visible after cultivation as a clear intact agar rounded by an opaque continuous bacterial coat.

In this communication, we attempted to compare the effect of negative corona discharge at various conditions on a set of particular microorganisms.

Experimental Part

Plasma Generation

NTP was generated using the previously described[18] simple apparatus of an open air type. Briefly, the negative point-to-plane corona discharge was generated on the point electrode represented by the tip of a syringe needle, connected to the source of direct current high voltage. The plane anode, connected to the positive pole of the source, was realized by the surface of a semisolid ion-conducting cultivation medium. The distance of the point electrode from the anode surface was set by a micrometer screw. The used source HT 2103 (Utes Brno, Czech Republic) made it possible to set a variable voltage up to 10 kV and current up to 0.5 mA. The experimental arrangement is depicted schematically in Figure 1. Further experimental parameters are described in following paragraphs.

Microbial Cultures

The microorganisms under study included a yeast *Candida albicans* and bacteria *Deinococcus radiodurans*, *Enterobacter aerogenes*, *Enterococcus faecium*, *E. coli, Geobacillus stearothermophilus*, *Neisseria sicca*, *Staphylococcus epidermidis, Stenotrophomonas maltophilia,* and *Streptococcus sanguinis*, obtained from the Czech

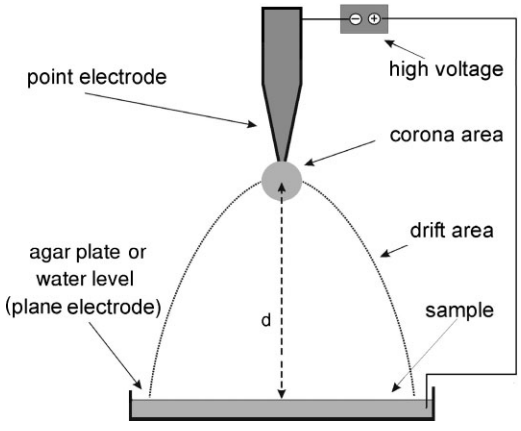

Figure 1. Scheme of the apparatus for generation of low temperature plasma by negative corona discharge.

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Collection of Microorganisms, Brno (CCM). All strains were propagated by cultivation in a liquid nutrient broth. The cells were suspended in sterile water with the addition of 10% glycerol as a preservative. Their bacterial concentration, expressed as number of colony forming units (cfu) related to cm^2 of agar plate or to mL of suspension was determined by dilution, plating, and counting of colonies. These stock suspensions were stored in a fridge until further diluted and either inoculated onto experimental plates or used as a suspension. In the case of *Geobacillus stearothermophilus*, the fresh culture without freezing was used, however, the presence of spores cannot be excluded even in the vegetative culture.

Sporulated forms of *Geobacillus stearothermophilus* were prepared by maintaining a stock suspension for 14 days at 20 °C. This led to an incomplete spontaneous sporulation. Immediately before the exposure, the suspension was warmed up to 75 °C for 15 min. This treatment inactivated all vegetative forms of the bacteria and the spores were at the same time activated to germination to the vegetative forms. Since this transition takes several hours, the inoculum could be considered to contain only the spores throughout the exposure period. Prior to exposure, the concentration of spores was determined by colony counting and the stock suspension diluted to the concentration of ca.100 cfu $\cdot$ cm^{-2}.

Exposition and Cultivation on Agar Plates

Aliquots (1 μL) of bacterial suspension at ca. 10^6 cfu $\cdot$ cm^{-2} were plated onto the whole surface of the semisolid Mueller-Hinton Nutrient Agar (Lab M, Inc.) in a Petri dish. For the yeast, the Sabouraud Dextrose Agar (Oxoid) was used. Preliminary experiments were also performed on a common blood agar and Endo agar. Immediately after absorption of the inoculum, all microorganisms were exposed to the corona discharge with an initial current of 0.05 mA (see Preliminary Experiments) at a variable inter-electrode distance of 2, 4, 6, and 10 mm for 1, 2, 4, 8, and 16 min. Bacterial spores were inoculated at the low concentration of ca.100 cfu $\cdot$ cm^{-2} and exposed at the same inter-electrode distances for 2, 4, 8, 16, and 32 min. To compare spores with vegetative cells, the nonsporulating *Staphylococcus epidermidis* was inoculated and exposed as spores. After exposure, all plates were cultivated at 37 °C overnight. All expositions were performed under laminar flow of HEPA-filtered air to prevent the airborne contamination. The ambient conditions were controlled by an air-conditioning of the laboratory.

Exposition of Liquid Suspensions

The stock bacterial cultures under study were adjusted to the initial concentrations from ca. 5×10^3–5×10^4 (low concentration) to 10^7–10^8 cfu $\cdot$ mL^{-1} (high concentration). Aliquots of 0.5 mL were pipetted into the wells of a dot plate and grounded using the immersed platinum wire, so that the surface of liquid became the plane electrode. The suspensions were exposed to negative corona discharge with a current of 0.05 mA at an inter-electrode distance of 3 mm for various time intervals and evaluated as described in the following paragraph.

Evaluation of Bacterial Cultures

The state of the cultures was assessed quantitatively. On the agar surface, the inhibition zones where a complete growth inhibition took place were extrapolated to an elliptic shape, their two diameters measured and the area S calculated. Following exposition in water suspension, the content of each well was diluted, spread onto the surface of Mueller-Hinton agar, cultivated at 37 °C overnight, the number of colonies were counted and the concentration of surviving microbes was calculated.

Results

Preliminary Experiments

The discharge could affect the composition of the media and make it potentially unsuitable for the growth. To exclude this adverse effect, the cultures were inoculated onto media previously exposed to the corona discharge. There were found no differences between the growth on exposed and unexposed media if the exposure was shorter than ca. 60 min. After longer exposures, the growth of bacteria was limited probably due to a gradual medium dehydration. Any considerable heating of the surface, which should be apparent as agar melting above ca. 40 °C, was not observed.

Effects caused by particle streaming (ion wind) have been observed on agar media. The ion drift carries with it considerable amount of air from the point electrode to the plane electrode and the streaming air gradually dehydrates and deforms the agar. The surface of the agar therefore corrugates to form a pit whose depth increases with progressing exposure. This effect was more conspicuous at lower initial distances of the needle tip from the agar surface. Due to the bending, the surface of the agar (i.e., the plane electrode) moved away from the point electrode; this caused a slight drop in the current passing through the discharge at a constant voltage. This effect was not corrected, as it was the same for all compared expositions. In the liquid, the ion wind causes its vigorous mixing and undulation, so that the discharge current slightly oscillates.

The appearance of inhibition zones on cultivation plates is demonstrated in Figure 2. It differs in dependence on the

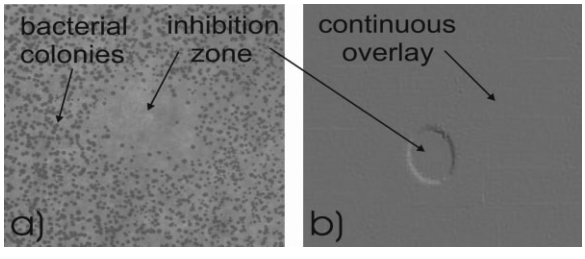

Figure 2. Typical appearance of inhibition zones at low (a) and high (b) concentration of inoculum. Note the isolated colonies and diffuse edges of the zone at left and continual growth and sharp edges of the zone at right.

concentration of inoculum: if the high concentration was used, the continual coat of bacteria and sharply demarcated zones appeared [see Figure 2(b)]. At high concentrations, the zone areas are independent on the concentration of inoculated bacteria, as verified on *Enterobacter aerogenes* and *Neisseria sicca*: stock suspensions and those diluted to the concentrations of 10^5 and 10^4 cfu · cm^{-2} were inoculated and exposed at $d = 4$ mm and $t = 8$ min. The size of the resulting inhibitory zones remained the same, being 15 and 37 mm^2 for *Enterobacter aerogenes* and *Neisseria sicca*, respectively. However, the cultures further diluted to a low concentration of ca. 100 cfu · cm^{-2} yielded cultures with isolated colonies, in which significantly larger but not sharply demarcated inhibition zones with diffuse edges developed [see Figure 2(a)].

Effects on Vegetative Forms

In all cases, the high concentration of ca.10^6 cfu · cm^{-2} was inoculated. The inhibition zone areas S obtained for *Candida albicans* exposed to the corona discharge on the agar surface for different exposition time t at a variable inter-electrode distance d is shown as an example in Figure 3. As seen from this graph, the inhibition zone size increased with exposure time, but the reciprocal proportion of zone size on the inter-electrode distance was irregular. These anomalies could be partly due to an error in measuring the inhibition zone diameters, that was estimated from triplicate measurement to be of ±0.5 mm. Similar graphs were obtained also for other microorganisms, differing only in somewhat lower S values and slight anomalies in the S/d dependence; they are not presented here in details. Instead of it, the largest observed zones S_{max} occurring in all cases after 16 min expositions but at various electrode distances were drawn into results presented in Table 1. At exposures up to

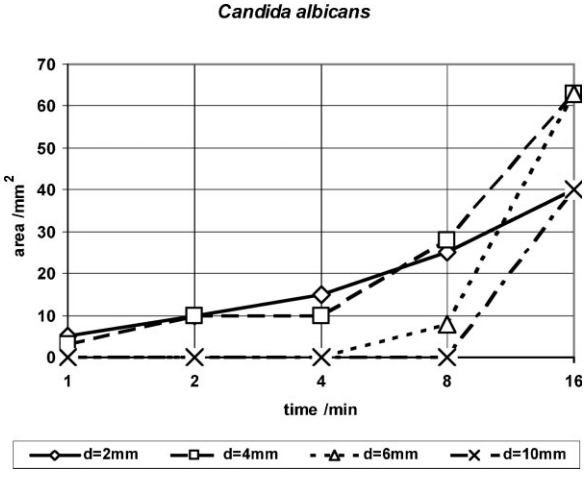

Figure 3. Dependence of inhibition zone area S on exposition time t and electrode distance d for *Candida albicans* inoculated in high concentration of 10^6 cfu · cm^{-2}.

Table 1. Maximal inhibition zone areas S_{max} obtained for various microorganisms after the 16 min exposition at various interelectrode distances.

Microorganism	S_{max} / mm^2
Candida albicans	63
Deinococcus radiodurans	56
Staphylococcus epidermidis	56
Neisseria sicca	50
Escherichia coli	50
Stenotrophomonas maltophilia	44
Enterococcus faecium	38
Streptococcus sanguinis	38
Enterobacter aerogenes	20
Geobacillus stearothermophilus vegetative form	20

2 min, all microbes displayed negligible zones except of *Stenotrophomonas maltophilia*, where $S = 10$ mm^2 was observed already after 1 min.

Effect on Bacterial Spores

In contrast to vegetative forms, no marked inhibition of *Geobacillus stearothermophilus* spores inoculated at a high concentration of ca.10^6 cfu · cm^{-2} was observed for exposure times shorter than 30 min. Although inhibition zones were formed at longer exposure times, it was not clear if they were due to an actual spore inhibition or to a partial degradation of the culture medium at very long exposures. The following results were therefore obtained for a low spore concentration and longer exposition times and compared with the nonsporulating *Staphylococcus epidermidis* inoculated at the same low concentration of 80 cfu · cm^{-2}. The resulting diffuse inhibition zones were measured and their areas S are given in Figures 4 and 5.

Exposition of Liquid Suspensions

The concentrations of particular microbes after various exposition times are plotted in graphs on Figures 6–8. Note the different time intervals on x-axes, respecting the different sensitivity of microbes: *E. coli* was inactivated after 75–120 s, *Staphylococcus epidermidis* after 4–5 min, whereas *Candida albicans* needed up to 30 min.

Discussion

Effects on Vegetative Forms

As seen from the Figure 3, the inhibition zone size increased regularly with exposure time, but the expected reciprocal

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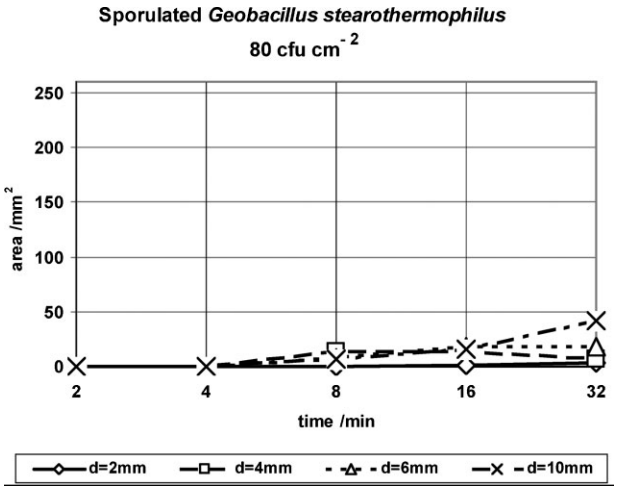

Figure 4. Effect of negative corona on low concentration of sporulated *Geobacillus stearothermophilus*.

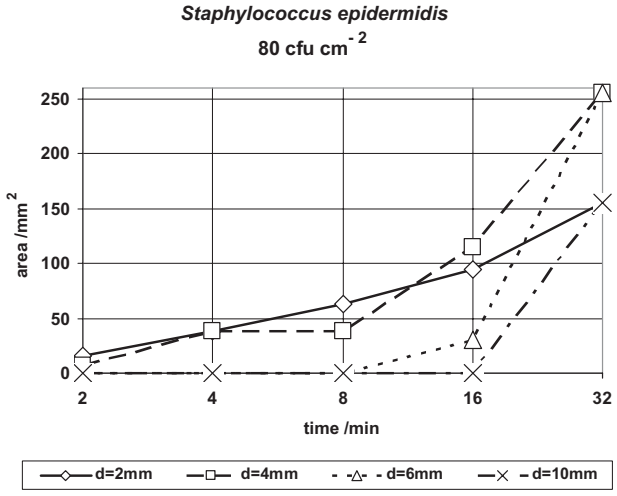

Figure 5. Effect of negative corona on low concentration of *Staphylococcus epidermidis*.

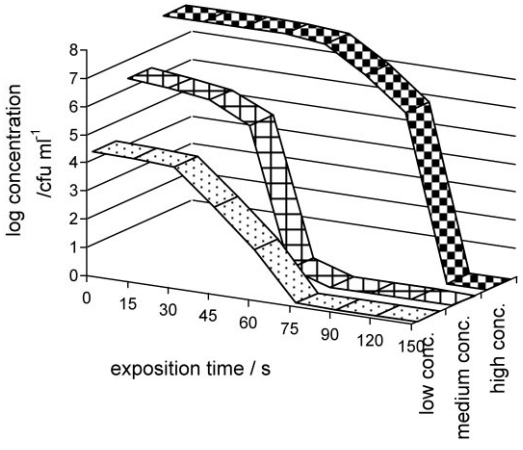

Figure 6. Effect of negative corona on aqueous suspension of *E. coli*. Note that intervals on time axes differ in Figures 6, 7, and 8.

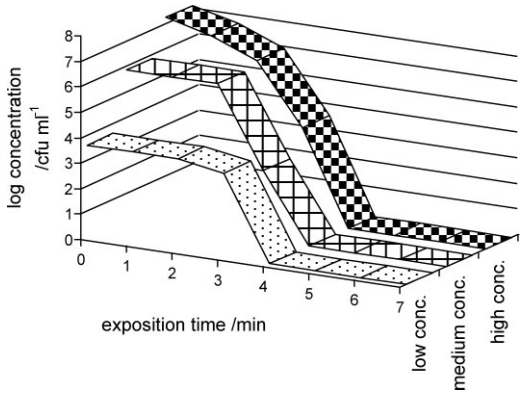

Figure 7. Effect of negative corona on aqueous suspension of *Staphylococcus epidermidis*. Note that intervals on time axes differ in Figures 6, 7, and 8.

proportion of zone size on the inter-electrode distance was mostly irregular. The first possible cause of this may be the poor accuracy in zone area measurement. Secondly, it may be caused by the properties of diffuse zone (drift area) of the discharge, described by Warburg's law: the concentration of reactive particles in the diffuse conus decreases with increasing distance d; at he same time, however, this conus constricts with decreasing d affecting a lesser area of the sample. Hence, the resulting inhibition area is a resultant of both these contradictory influences.

At large inter-electrode distances and short exposures, the microbicidal effect of the discharge was negligible except for *Stenotrophomonas maltophilia*, which exhibited marked zones even under these conditions. On the other hand, the largest zones were obtained after long exposures regardless of the inter-electrode distance.

The sensitivities of all microorganisms under study to the microbicidal effect of the corona discharge appeared to be of the same order. Despite the small differences, it was easy to

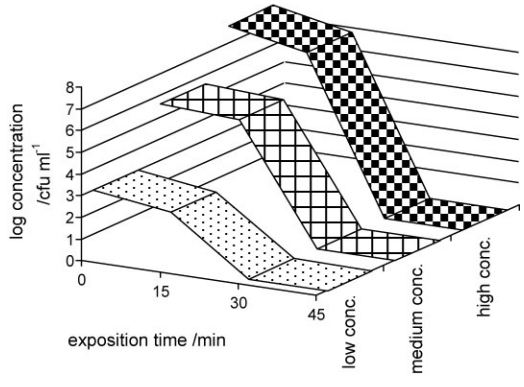

Figure 8. Effect of negative corona on aqueous suspension of *Candida albicans*. Note that intervals on time axes differ in Figures 6, 7, and 8.

arrange the microorganism under investigation into order of decreasing sensitivity using the largest area of the inhibition zone observed after a 16 min exposure as the sensitivity criterion. Surprisingly, no significant differences were found between Gram-positive and Gram-negative bacteria, or between cocci and rods; e.g., the Gram-positive *Geobacillus stearothermophilus* possesses the same resistance as the Gram-negative *Enterobacter aerogenes*. The exceptional sensitivity of *Stenotrophomonas maltophilia* at low exposure times could be of potential practical significance since a similar sensitivity can be presumed also in its close relative, the pathogenic *Pseudomonas aeruginosa*.

An interesting feature is also the "ordinary" sensitivity of *Deinococcus radiodurans* to NTP, which was observed in our previous work[17] and confirmed in ref.[12] The extreme resistivity of this bacterium to electromagnetic radiation, acting mainly on chromosomal DNA, is caused by the extraordinary arrangement of its genetic information containing backup copies of genome in four independent DNA molecules.[19,20] As previously shown by electron microscopy (see e.g.,[21,22]), the bactericidal effect of plasma consists mostly in bacterial wall and/or membrane damage, which leads to bacterial disruption and lysis, although effects on other parts of bacteria (e.g., DNA) cannot be excluded. Nevertheless, *Deinococcus radiodurans* has no special defense against lysis mediated mostly by reactive particles rather than radiation. This finding supports the hypothesis of the low importance of UV radiation in the decontamination by the corona discharge.

Although there are a few studies using methods comparable with our study, the one of Noyce and Hughes[13] should be mentioned: under atmosphere of nitrogen, the reduction of *E. coli* was observed, but the lethality did not exceed 98% even after 30 min exposure.

Effect on Bacterial Spores

The sporulating bacterium *Geobacillus stearothermophilus* is used routinely for the validation of sterilization procedures. As expected, its spores displayed a markedly lower sensitivity to the action of the plasma generated by corona discharge when compared with the vegetative forms. A significant microbicidal effect was observed only after longer exposures and when lower initial spore concentration was used. The spore inhibition appeared after an 8 min exposure at the earliest. This corresponds roughly to previous results of Akishev et al.,[7] where the inhibition of *Bacillus subtilis* spores was observed after 7 min of exposition to atmospheric pressure plasma jet in air. Zones of comparable size of the order of 10 mm^2 appeared with the vegetative forms after a 2–16 min exposure whereas a 16–32 min exposure was needed with the spores. Hence, the efficiency of action of the discharge on spores can be roughly estimated to be 4- to 8-fold less than with vegetative forms.

Exposition of Liquid Suspensions

The action of negative corona discharge causes the complete sterilization of bacteria in 0.5 ml liquid samples within minutes. The sensitivity of tested microbes, however, differs significantly: the Gram-negative bacterium was killed after 2 min of exposition, whereas for the Gram-positive bacterium a 4 min exposition was necessary for the same effect. This may be explained by the different cell wall structure, which is thin and fragile in Gram-negatives and strong in Gram-positives. It remains a question, why this fact did not influence the sensitivity of various bacteria on agar surface.

Surprisingly, the 30 min exposition was necessary to kill the yeast *Candida albicans*. This trend is in strong opposition to the results achieved in thin film on the surface of water-rich semisolid media, where the yeast appeared to be the most sensitive organism. Maybe this discrepancy is caused by the different efficiency of mixing the bulk volume of liquid sample by the ion wind, which is less efficient for the comparatively large and heavy yeasts than for tiny bacterial cells. The active plasma particles apparently act in the thin surface layer of the water, so that yeast cells should be protected in the deep, where secondary stable particles with different bactericidal properties (as H_2O_2) may also be formed. Except this speculation, we have no reasonable explanation of this effect.

Conclusion

The bactericidal effect of negative corona discharge on microbes spread on agar surface is of comparable magnitude for yeast, Gram-positive, and Gram-negative bacteria of various shapes including highly resistant *Deinococcus radiodurans*. Bacterial spores are of almost one order less susceptible than vegetative forms. Bacteria in liquid suspension are completely inactivated within the 5 min of exposition, whereas the exposition up to 30 min is necessary for the yeasts.

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