LOW TEMPERATURE DESTRUCTION OF BACTERIA *BACILLUS STEAROTHERMOPHILUS* BY WEAKLY IONIZED OXYGEN PLASMA

NIZKOTEMPERATURNO UNIČEVANJE BAKTERIJ *BACILLUS STEAROTHERMOPHILUS* S ŠIBKO IONIZIRANO KISIKOVO PLAZMO

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Weakly ionized plasma created in oxygen at low pressure was applied in order to destruct bacteria *Bacillus stearothermophilus*. Plasma was created in an inductively coupled radiofrequency discharge. The kinetic temperature of neutral gas was kept close to room temperature in order to prevent substantial thermal damage. Plasma parameters depended on the pressure of oxygen in the reactor. The density of charged particles was decreasing with increasing pressure while the density of neutral oxygen atoms exhibited an opposite behavior. Plate count technique as well as scanning electron microscopy was used to monitor the bacterial destruction. Experiments performed at different pressures indicate that the neutral O atoms are major reactants causing etching of organic material, while ultra violet radiation from plasma is responsible for rapid deactivation in the first few seconds of plasma treatment.

Keywords: sterilization, oxygen plasma, bacteria, *Bacillus stearothermophilus,* plate count technique, scanning electron microscopy

Nizkotlačno kisikovo plazmo smo uporabili za uničevanje bakterij *Bacillus stearothermophilus*. Plazmo smo vzbujali v induktivno sklopljeni visokofrekvenčni razelektritvi. Kinetična temperatura nevtralnega plina je bila blizu sobni, s čimer smo se izognili neželenim termičnim efektom. Plazemski parametri so bili odvisni od tlaka kisika v reaktorju. Gostota nabitih delcev je z naraščajočim tlakom padala, medtem ko je gostota nevtralnih kisikovih atomov naraščala. Za določanje števila preživelih bakterij smo uporabili inkubacijo na krvnem agarju s štetjem kolonij, za določanje poškodb pa vrstično elektronsko mikroskopijo. Raziskave, opravljene pri različnih tlakih, so pokazale, da je jedkanje organskega materiala, ki sestavlja bakterije, predvsem posledica interakcije nevtralnih kisikovih atomov. Hiter padec števila preživelih bakterij v prvih nekaj sekundah plazemske obdelave pa smo pripisali vplivu ultravijoličnega sevanja.

Ključne besede: sterilizacija, kisikova plazma, bakterije, *Bacillus stearothermophilus*, krvni agar, vrstična elektronska mikroskopija

1 INTRODUCTION

Polymer materials applied in biology and medicine should be sterile. This is especially the case for body implants where the requirements for sterility are sever. Several techniques have been developed to assure sterility of materials and devices. They include poisonous chemical reagents, a variety of energetic beams and heat treatments. All these techniques have advantages and drawbacks. In practice, heat treatment using both dry and wet atmosphere is particularly popular since the equipment is rather inexpensive and easy to use. Samples are mounted into an appropriate chamber and heated to elevated temperatures. Dry heating usually requires higher temperatures and/or longer treatment times comparing to wet treatment. Water vapour is an excellent medium for rapid heat transfer from a boiler to the samples. The device is called an autoclave and is widely used in common practice.

A small disadvantage of heat treatment is a simple fact that many materials do not stand prolonged heating. This is especially the case for many polymers since structural modifications may occur. Many body implants are made from or contain polymer components so the problem is pretty severe. In such cases autoclaving is not the right sterilization method and other methods should be applied. The most straight-forward method is application of poisonous liquids or gases. There are a handful of such reagents such as ethylene- oxide, fluorine and chlorine. No bacteria or spores can stand prolonged treatment with such poisonous compounds. A small problem arises from the simple fact that such reagents are also poisonous for humans. Such sterilization should be performed using strict protocols, and accidents are not uncommon. It is especially difficult to remove remains of reagents as well as death bacteria that may remain toxic.

Energetic beams are increasingly popular in sterilization of delicate materials. Energetic electrons, ions, γ-rays and hard UV radiation can be applied. The procedure is effective, but unsuitable for sterilization of objects with complex shape since the energetic beams do not always reach gaps and other small features. The sterilization of implants is therefore not a trivial task and many researchers worldwide attempt to develop more

reliable methods. One of them is application of cold weakly ionized gaseous plasma.[1–16] The present paper addresses plasma sterilization of materials contaminated with heat resistive bacteria *Bacillus stearothermophilus*.

2 EXPERIMENTAL

Destruction of bacteria *Bacillus stearothermophilus* was performed in a small plasma reactor which has been described to details previously.[1–11] Plasma has been well-characterized with a Langmuir probe and a catalytic probe.[17–23] Plasma parameters have been measured in a wide range of pressures from 10 Pa to 200 Pa.[28] The density of charged particles and the density of neutral oxygen atoms at the pressures at which sterilization was performed are summarized in **Table 1**.

Table 1: Two most important plasma parameters (ion and neutral atom densities) at three selected pressures in the vacuum chamber
Tabela 1: Dva najpomembnejša plazemska parametra (gostota ionov in nevtralnih kisikovih atomov) pri treh izbranih tlakih v vakuumski posodi

Pressure	Charged particle density	Neutral O atom density
30 Pa	$2.5 \cdot 10^{16}$ m^{-3}	$1.9 \cdot 10^{21}$ m^{-3}
75 Pa	$8 \cdot 10^{15}$ m^{-3}	$3.2 \cdot 10^{21}$ m^{-3}
150 Pa	$4 \cdot 10^{15}$ m^{-3}	$4.6 \cdot 10^{21}$ m^{-3}

The samples of *Bacillus stearothermophilus* were prepared according to the standard procedure. The bacteria were first incubated and then rinsed thoroughly in order to remove traces of nutrition in the incubating suspension. The initial concentration of bacteria in suspension was determined by a standard plate count technique and was about 8×10^8 cells per ml. A drop of water containing bacteria with the volume of 100 μL was deposited onto carriers. We used standard well-polished silicon substrates. The substrates were pre-cleaned by chemical methods and exposed to oxygen plasma for few seconds in order to remove any possible traces of impurities that may prevent an even distribution of the water drop. The substrates were dried at ambient temperature and after water evaporation a rather uniform film of bacteria was created. The number of bacteria per carrier was about 8×10^6 cells (vegetative form only).

Carriers with bacteria were exposed to plasma for various times and at three different pressures of (30, 75 and 150) Pa. The corresponding plasma parameters are presented in **Table 1**. After each treatment the bacteria were washes by distilled water, diluted and applied onto agar plates. They were incubated for about 24 h so that visible colonies appeared on the agar plate. The number of visible colonies was measures and, taking into account the dilution ratio, the number of survived bacteria on the substrates was calculated.

Selected samples were mounted into a scanning electron microscope to determine any visible changes of bacteria. We used a high resolution scanning electron microscope (SEM) with a field emission source of electrons Carl Zeiss Supra 35. The kinetic energy of primary electron beam was adjusted to1 keV. No coating was applied onto the samples prior to SEM analyses.

3 RESULTS

Results of systematic measurements of the survival rate are summarized in **Figure 1** and **Figure 2**. **Figure 1** represents results at the oxygen pressure of 75 Pa while **Figure 2** is for two different pressures: 30 Pa and 150 Pa. A pretty sharp drop of the live bacteria is observed after plasma treatment for a short time and the concen-
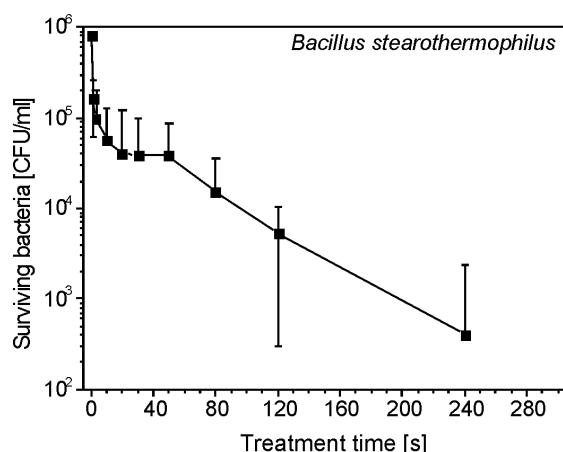

Figure 1: Survival curve of *Bacillus stearothermophilus* by PCT. The graph represents CFUs of *Bacillus stearothermophilus* versus oxygen plasma treatment time at 75 Pa.
Slika 1: Število bakterij *Bacillus stearothermophilus*, ki smo jih ugotovili z metodo nanosa na plošče krvnega agarja. Slika predstavlja prešteto število enot, ki sestavljajo kolonije, v odvisnosti od časa plazemske obdelave pri tlaku 75 Pa.
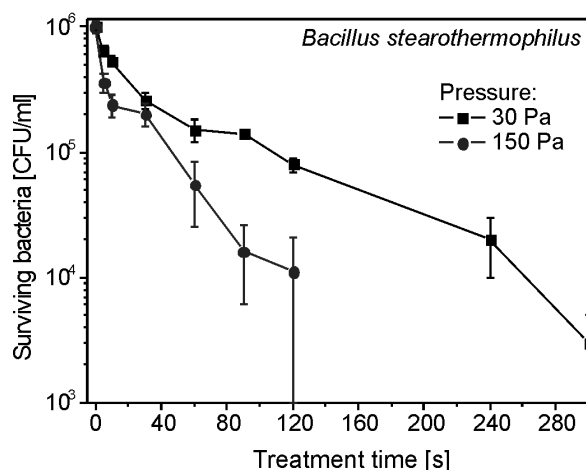
Figure 2: *Survival curves of Bacillus stearothermophilus by PCT.* The graph represents CFUs of *Bacillus stearothermophilus* vs. treatment time by oxygen plasma at pressures of 30 Pa and 150 Pa.
Slika 2: Število bakterij *Bacillus stearothermophilus*, ki smo jih ugotovili z metodo nanosa na plošče krvnega agarja. Slika predstavlja prešteto število enot, ki sestavljajo kolonije, v odvisnosti od časa plazemske obdelave pri tlakih 30 Pa in 150 Pa.
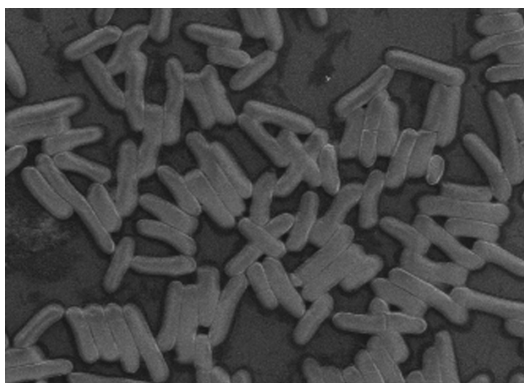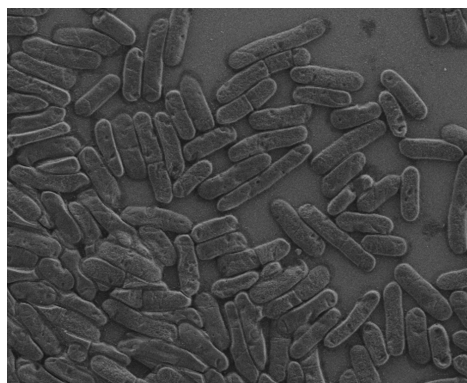
Figure 3: A SEM image of untreated bacteria
Slika 3: Posnetek neobdelanih bakterij

Figure 5: A SEM image of bacteria treated by oxygen plasma for 10 s
Slika 5: Posnetek bakterij, ki so bile obdelane s kisikovo plazmo 10 s

tration was decreasing monotonously with increasing oxygen plasma treatment time.

Selected samples were imaged by scanning electron microscopy. **Figure 3** represents a typical image of an untreated sample. The SEM image of a sample exposed to plasma for 3 s is presented in **Figure 4**. No visible changes are detected as compared to the untreated sample. **Figure 5** represents SEM image of a sample exposed to plasma for 10 s. Here, some modifications of the bacteria are observed, but the original shape is more or less preserved. The sample treated for 55 s is presented in **Figure 6**. The bacteria are now visibly damaged.

4 DISCUSSION

The results presented in **Figures 1 and 2** reveal interesting features. The initial number of survived bacteria drops significantly even for the shortest treatment time. Further on, a plateau is observed in the survival curves, and for prolonged treatment the number of survived bacteria is very small, but still measurable. The appearance of the plateau can be explained by different destruction mechanisms. Oxygen plasma is a rich source of particles that may be harmful to bacteria. The particles include excited oxygen molecules, atoms in

the ground state, metastable excited atoms, positively charged ions, negatively charged ions and photons emitted at relaxation of excited states by electrical dipole radiation.[24–27] All these particles may react with bacteria. It has been shown by numerous authors that organic material is slowly etched during treatment with oxygen plasma.[29–33] The etching has been attributed to chemical interaction between neutral oxygen atoms that abound in oxygen plasma and organic materials. The etching rate depends on plasma parameters as well as on the substrate temperature. At low temperature, the rate is often of the order of 10^{-4}.[29–31] This value increases as the temperature is increasing.

The sharp decrease of the number of survived bacteria observed in the first few seconds of plasma treatment (**Figures 1 and 2**) can be hardly attributed to etching by neutral oxygen atoms. The sample temperature remains close to room temperature for this short period of plasma treatment so the reaction probability remains low. Also, the SEM image after 3 s of plasma treatment does not reveal any visible modification of the bacterial cell wall (**Figure 4**). The sharp drop in the number of survived bacteria in first few seconds of treatment by oxygen plasma should be therefore attributed to other mechanisms.
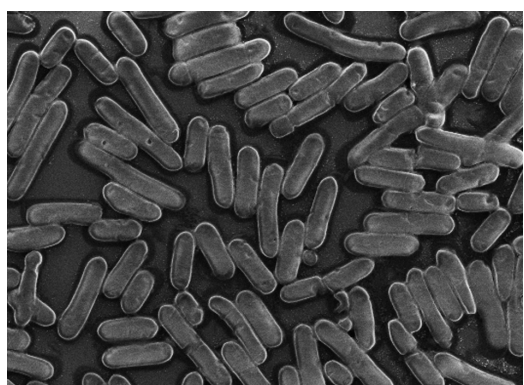
Figure 4: A SEM image of bacteria treated by oxygen plasma for 3 s
Slika 4: Posnetek bakterij, ki so bile obdelane s kisikovo plazmo 3 s
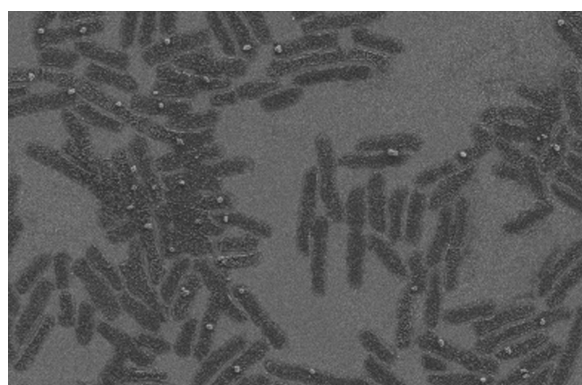
Figure 6: A SEM image of bacteria treated by oxygen plasma for 55 s
Slika 6: Posnetek bakterij, ki so bile obdelane s kisikovo plazmo 55 s

Excited oxygen atoms are definitely more aggressive against organic materials, but they cause similar damage as neutral oxygen atoms in the ground state. The same applies for metastable molecules that are expected to be present in oxygen plasma in a pretty high concentration. Negatively charged oxygen ions are unlikely to reach the surface of the samples as a thin sheath is always formed between the unperturbed plasma and the sample surface. A potential drop within this sheath repels negatively charged particles so that the flux onto the surface is negligible. Positively charged molecules and ions, on the other hand, are accelerated across the sheath and bombard the surface with the kinetic energy obtained within the sheath. The voltage drop within the sheath depends on the external biasing and could be pretty large in capacitively coupled plasma. In our case, however, we use inductively coupled plasma and the samples are not connected to an electrode. The samples are just kept at the floating potential. The difference between the plasma and floating potentials depends on electron temperature. In our case, this temperature is only a few 10 000 K, so the potential difference is of the order of 10 V. Assuming a collision-less sheath, the ions gain energy of the order of 10 eV within the sheath. The penetration depth of such ions in a solid material is only of the order of a nm, so they are unlikely to cause any damage to vital parts of bacteria. The rapid drop in the number of survived bacteria observed in **Figure 1 and 2** therefore cannot be explained by the action of ions.

The only particles left are photons. Plasma is a rich source of radiation in the range from infra-red to ultraviolet light. The radiation is best observed by optical emission spectroscopy.[35–41] The transitions between excited and ground states as well as between higher and lower excited states may occur in oxygen molecules and atoms as well as impurities that are always presented in gaseous plasma. Neutral oxygen molecules are poor light emitters since the transition by electrical dipole radiation is prohibited by laws of quantum mechanics. Charged molecules are scarce in our plasma (see **Table 1**) so the emission of light quanta is easily neglected. Atoms, on the other hand, abound in our plasma as shown in **Table 1**. Some atoms are excited to highly excited states that radiate at different wavelengths. The photons have energies either in the far UV (approximately 10 eV) or in visible range (mostly at about 2 eV). The visible light in the red part of spectrum is obviously not lethal to bacteria so they can be excluded from discussion on killing mechanisms. Hard UV radiation, on the other hand, is lethal for bacteria, but the concentration of atoms in highly excited states that produce them is pretty low (unfortunately very little work has been performed on quantitative determination of the radiation from this source). The rapid deactivation of bacteria as observed in **Figures 1 and 2** can be therefore only partly attributed to radiation from oxygen particles.

Plasma often contains impurities. The main impurity in simple vacuum systems is water vapour. Since the ultimate pressure in our system is few pascals, the concentration of water in the chamber filled with oxygen is few percent. Water molecules are quickly dissociated to OH and H radicals. Both are excellent sources of UV radiation. The Lyman series of H atoms is all in UV range, while OH molecules have rich transitions in the near UV region.[42] The UV photons destroy the DNK of bacteria and are therefore likely to contribute significantly to deactivation of bacteria. The quick drop in the survival curves in first few seconds of plasma treatment (**Figures 1 and 2**) is therefore likely to be explained by absorption of UV radiation. UV radiation does not cause a visible damage to organic materials so this mechanism explains the preserved shape of bacteria observed in **Figure 4**.

Once many bacteria are damaged by UV radiation, the bacteria in agglomerates are only preserved. While it was difficult to observe such agglomerates they are definitely present. As long as bacteria are sheltered in agglomerates their deactivation by UV radiation is not likely to occur. They have to wait for other mechanisms to become effective. Such mechanism is chemical etching predominantly by neutral oxygen atoms. **Figure 5** represents a SEM image of bacteria after 10 s. Visible changes in morphology can be observed. The effect is even much more pronounced after 55 s of plasma treatment. The corresponding SEM image is presented in **Figure 6**. In fact, bacteria presented in Figure 6 are really badly damaged. Only ashes are left as remains of bacteria. **Figure 6** also reveals small but perfectly visible spheres that are left after ashing process induced by oxygen plasma treatment. The origin of the spheres is unknown and any discussion on this phenomenon is beyond the scope of this paper.

There is a discrepancy between results presented in **Figures 1 and 6**. Namely, **Figure 6** reveals only ashes on the substrates while the results presented in **Figure 1** clearly show that not all bacteria are damaged lethal. A possible explanation of this discrepancy may be connected to appearance of agglomerates. Since the number of SEM images taken at these experiments was limited, we might have missed such agglomerates. Namely, the bacteria presented in **Figure 6** are definitely damaged so badly that they cannot be revitalized.

It is worth discussing the effect of pressure on the bacteria deactivation. Comparison of results presented in **Figure 2** indicates that the deactivation is much faster at high pressure. At the treatment time of 120 s, for instance, the number of survived bacteria at 30 Pa is about 80 000, while at 150 Pa it is only little more than 10 000 – almost an order of magnitude smaller. This pretty big difference may be explained taking into account different plasma parameters at different pressures as shown in **Table 1**. The density of charged particles at 30 Pa is almost an order of magnitude larger

than at 150 Pa. If bacteria were deactivated by charged particles one would expect higher deactivation at lower pressure. The results show right the opposite effect. The effect is explained by interaction of neutral oxygen atoms with bacteria. The density of oxygen atoms at 150 Pa is more than double of the value measured at 30 Pa. This result, in combination with the results presented in **Figure 2**, indicate that neutral oxygen atoms play a dominant role in deactivation of bacteria in the second phase, i.e. after destruction by UV radiation. Any quantification of the destruction probability versus the density of atoms is probably not justified for many reasons including the experimental error (both in determination of the O density and in determination of the number of survived bacteria), the unknown rate of agglomeration, and the fact that the destruction rate is not linear with the flux of neutral oxygen atoms onto the surface of bacteria. The temperature effects may not be negligible, too. While any substantial heating can be excluded for first few seconds of plasma treatment the thermal effects may not be negligible after prolonged treatment. Namely, although the neutral gas kinetic temperature is close to room temperature, exothermic reactions take place on the substrate surface due to interaction of various plasma particles with the solid material. The reactions include heterogeneous surface recombination of neutral oxygen atoms, neutralization of charged particles, weak bombardment with positive ions and de-excitation of metastable atoms and molecules. The reaction rates are, unfortunately, not known so any estimation of the bacterial temperature versus the plasma treatment time is beyond the scope of the paper. Unfortunately, the experimental system in present configuration does not allow for measuring the temperature.

Finally it is interesting that complete sterilization was not obtained even after 5 minutes of plasma treatment. This effect is difficult to explain by agglomerates since **Figure 6** shows that even after 55 s the bacteria in monolayer are ashed. The facts observed in **Figures 1 and 2** may be explained with un-careful handling of bacteria. Namely, the plate count technique applied for determination of the number of bacteria is definitely sensitive to contamination. If foreign bacteria are presented in the experimental room they would add to the measured values obtained by this technique. Plate count technique unfortunately does not distinguish between bacteria used for the experiments and foreign bacteria that might have been present in the experimental room.

5 CONCLUSION

A method for sterilization of substrates contaminated with bacteria *Bacillus stearothermophilus* was presented. The method is based on application of weakly ionized oxygen plasma. Particles created in oxygen plasma interact with bacteria causing their degradation. The degradation was studied using the plate count technique. Rather rapid degradation was observed. The degradation mechanisms were discussed to some details. The combination of the results obtained by plate count technique and those obtained by scanning electron microscopy indicate that both destruction of bacterial DNA by absorption of UV radiation and chemical etching are responsible for deactivation or destruction of bacteria. In the first few seconds of plasma treatment the most likely mechanism is deactivation of bacterial DNK, and the chemical etching prevails thereafter. In any case it has been shown that plasma treatment is a promising technique for sterilization of delicate biomedical materials.

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