Inactivation of microorganisms in model biofilms by atmospheric pressure non-thermal plasma

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Résumé

Microorganisms have high ability to attach strongly to any surface and form biofilms. Biofouling can lead to corrosion of metallic constructions and decrease essentially the lifetime of wood and concrete facilities. Drawbacks of the traditional methods for microorganism inactivation and biofilm destroying stimulate a development of novel approaches to the protection against biofouling and biodamage processes. This paper reports fresh results on sterilization of microorganisms including biofilms with usage of non-thermal plasma.

Introduction

Protection of industrial facilities, devices, materials, etc against the biofouling and biodamage (including biocorrosion) is one of the great challenges of a modern science and industry. This problem is rather difficult to resolve since biofilm-forming microorganisms are highly resistant to biocides. For instance, sterilization of the waterworks requires too high dose of biocides that is harmful from environmental point of view. Additionally, water treatment by biocides takes too long time (more than twenty-four hours).

Here, we are dealing with so-called non-thermal plasma (NTP) sterilization of microorganisms including biofilms. NTP sterilization can lead to the eventual abandonment in usage of heat and the chemically aggressive, toxic and environmentally harmful liquid and gaseous agents. Our previous promising results on this topic are published in [1]. Present report contains results on atmospheric pressure NTP inactivation of microorganisms in model (monoculture of *Escherichia coli* and *Bacillus subtilis*) biofilms.

Biofilms are spatially and metabolically structured microbial communities within extracellular polymeric matrix at the phase interface. Biofilms distinguish themselves with a specific dynamics of the growth and substrate digestion modes, and are highly resistant to chemical biocides. A simple and convenient research model of a biofilm is pure culture of colonies grown on agar.

Types of microorganisms used

In our work we used two model biofilms generated by *E. coli* and *B. subtilis* monocultures. We studied the biofilms grown on the agar and on the surface of inert carriers (mild steel and polypropylene coupons) immersed in starvation (poor) medium. Biofilms grown on the agar are convenient to work with, but they are too simplified models of natural biofilms. Model biofilms grown on the surface of inert carriers are much closer to natural biofilms.

We treated so-called “young” biofilms grown overnight, and “old” biofilms grown for several days. The total number of microorganisms constituting *E.coli* biofilms grown in enriched medium reached 10^{11} CFU as early as overnight. As biofilms were getting old, their number did not change. But the biofilms increased in diameter, while their superficial density decreased (from 2.9×10^{9} CFU/mm^2 for a biofilm grown overnight to 3.6×10^{8} CFU/mm^2 for a three days old biofilm).

Results

After plasma processing, the biofilm grown overnight was found to be most susceptible, whereas the biofilm grown for three days was less susceptible. The decreased susceptibility of the “old” biofilm is likely associated with physical “ageing” of the biofilm microorganisms that leads to the cell division inhibition, whereas a pool of reserve cellular substances, as well as strength of the cell wall, increases. Besides, the “old” biofilms may contain a significant amount of the dead cells screening the living cells against plasma action.

Treating *E.coli* metal and plastic coupons with low-temperature plasma showed full inactivation of microorganisms of biofilms, with the majority of cells being killed within the first minute of the treatment (Fig.1). The biofilm grown for 3 days on a metal coupon appeared to be the least susceptible.

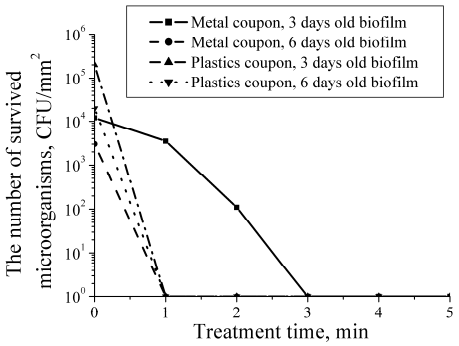

Fig. 1: The rate of survived *E.coli* age-varying biofilms grown on metal and plastic coupons.

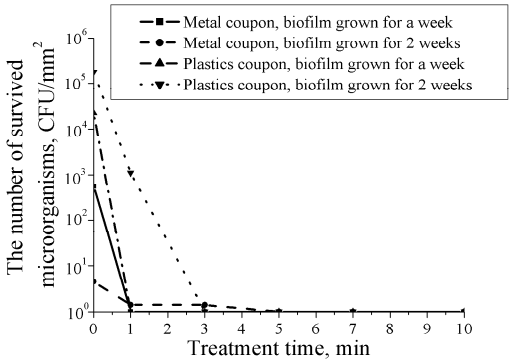

Fig. 2: The rate of survived *B.subtilis* age-varying biofilms grown on metal and plastic coupons.

Research on *B.subtilis* biofilms grown on both types of coupons has shown that these biofilms are more tolerant than *E.coli* biofilms (Fig.2). Although the initial titer of *B.subtilis* biofilms was lower than that of *E. coli* biofilms, they became inactivated in 3-5 minutes of the treatment. Besides, in contrast to *E.coli* biofilms, *green* (one week old) *B. subtilis* biofilms appeared more susceptible than two weeks old biofilms, probably due to the transition of some *B. subtilis* cells from vegetation to spores.

To clarify the mechanism of NTP-cell inactivation, we have done the experiments related to checking the integrity of some individual cell structures (cell wall and membrane) after plasma action. The method is based on measuring the number of free intracellular nucleotides released by a cell in response to the NTP exposure (2-10 min). Cells were precipitated by centrifugation (5810R *Eppendorf*; 12000 rpm) for 20 min. A concentration of supernatant nucleotides was determined from measurement of optical density of a liquid at $\lambda = 260$ nm by the spectrophotometer (*Shimadzu* UV-1700).

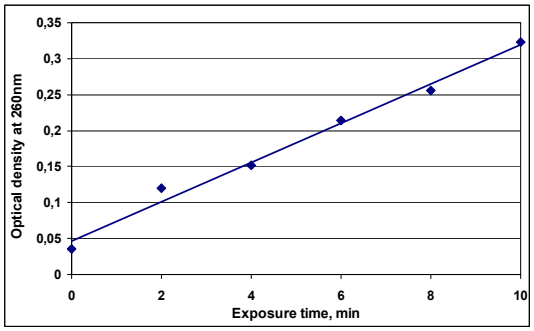

Fig. 3: The dynamics of free nucleotides concentration in supernatant vs NTP-exposure of *E.coli* cell suspension in the isotonic conditions.

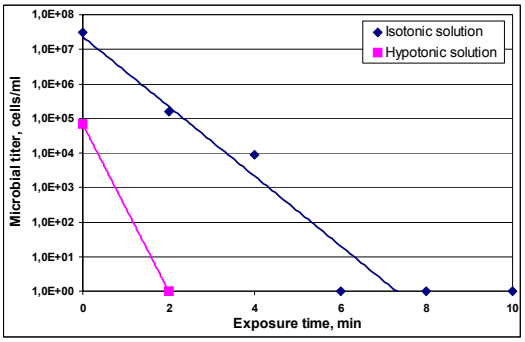

Fig. 4: Survivability of NTP-treated *E.coli* liquid culture that was incubated subsequently in the osmotic pressure-varying solutions.

Comments to Fig.3: Free nucleotides (mainly low-molecular-weight ones) appeared in the supernatant because of partial or complete degradation of both the cell wall and cell membrane. So cold plasma treatment brokes partially or fully the integrity of the cell wall and cell membrane and could kill thereby the cells.

Comments to Fig.4: The NTP-exposure results in partial or complete degradation of both the cell wall and cell membrane that leads to loosing the cell wall strength. After that, the cells with a depressed wall strength can be easily burst in hypotonic solution because of huge turgor pressure.

One can conclude that plasma sterilization procedure differs beneficially from conventional methods to control biofilms. No chemically aggressive reagents are required, and the plasma procedure takes short time. Owing to these features the NTP-procedure is expected to be widely applied in different areas where control and inhibition of biofilm growth are urgent (pipelines, surfaces of stone, wood and concrete buildings, etc).

References

[1] Yu. Akishev, M. Grushin, V. Karal'nik, A. Petryakov, N. Trushkin, et al., Pure Appl. Chem. **80** (2008) 1953.