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INACTIVATION OF POSSIBLE MICROMYCETE FOOD CONTAMINANTS USING THE LOW-TEMPERATURE PLASMA AND HYDROGEN PEROXIDE

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The inhibition effect of hydrogen peroxide aerosol, low-temperature plasma and their combinations has been studied on several micromycetes spores. The low-temperature plasma was generated in corona discharges in the open air apparatus with hydrogen peroxide aerosol. Micromycete spores were inoculated on the surface of agar plates, exposed solely to the hydrogen peroxide aerosol, corona discharge or their combination. After incubation the diameter of inhibition zone was measured. The solely positive corona discharge exhibits no inactivation effect, the solely negative corona discharge and solely hydrogen peroxide aerosol exhibit the inactivation effect, however their combinations exhibit to be much more effective. Low-temperature plasma and hydrogen peroxide aerosol present a possible alternative method of microbial decontamination of food, food packages or other thermolabile materials.

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INTRODUCTION

The fungal and other microbial contaminants may present a serious problem in medicine, food processing and other areas and those elimination is often desired in many area. One common method is the inactivation of microorganisms on surfaces by hydrogen peroxide, however due to preoxide high oxidative potential this method may be limited by the material applied on, ecological problems, safety manipulations with the chemicals or other problems (e.g. economical viewpoint). On the other side there are numerous of works describing the microbicidal effects of low-temperature plasma. The presented work studies the possibility of the use of combination of low-temperature plasma with the hydrogen peroxide for the inactivation of micromycetes (fungal) spores on surfaces what may help to reduce the adverse side effects. The biological effects of low temperature plasma, devoted mainly to the killing of prokaryotic bacteria may be found in reviews [1–4], various applications in human medicine in the article [5] and decontamination of the medical products may be found in [6]. The fungicidal effect of low-temperature plasma are presented less often and was mentioned e.g. in [7, 8] or in our recent work [9], where we have studied the inactivation of micromycetes spores in positive corona discharge. This work follows up our previously presented insights and describes the fungicidal effect of the synergy combination of low-temperature plasma generated in corona discharge and hydrogen peroxide aerosol. This combination leads to the decrease of the operation time up to several seconds and may be applicable for the treatment of food surface or food packages, preventing the mould overgrow and food spoilage, or for the decontamination of other thermolabile materials.

MATERIALS AND METHODS

The low temperature plasma was generated using the modification of current apparatus previously described in [10]. The modification allowed the addition of hydrogen peroxide aerosol into the atmosphere of applied discharge. The discharge burned between the point and grid electrodes. The distance between them is 6 mm. The point electrode was represented by the tip of a syringe needle situated vertically to the grid electrode. The grid consisted of stainless steel wire of diameter 0.25 mm forming the net with a mesh size of 0.8 cm. The discharge was stabilized by the connection of a serial resistance of 20 MΩ into the circuit. The discharge, similar to the pin-to-ring geometry, burns on the tip of the needle; on the closes parts of the wire burn secondary parasite discharges of inverse polarity but of lower intensity as it was observed by naked eye. Despite it, in this work, we call the discharge as positive corona if the polarity of the point electrode is positive and of the grid electrode is negative. Contrary, we call the discharge as negative corona if the polarity of the point electrode is negative and of the grid electrode is positive. For more details about the corona dis-

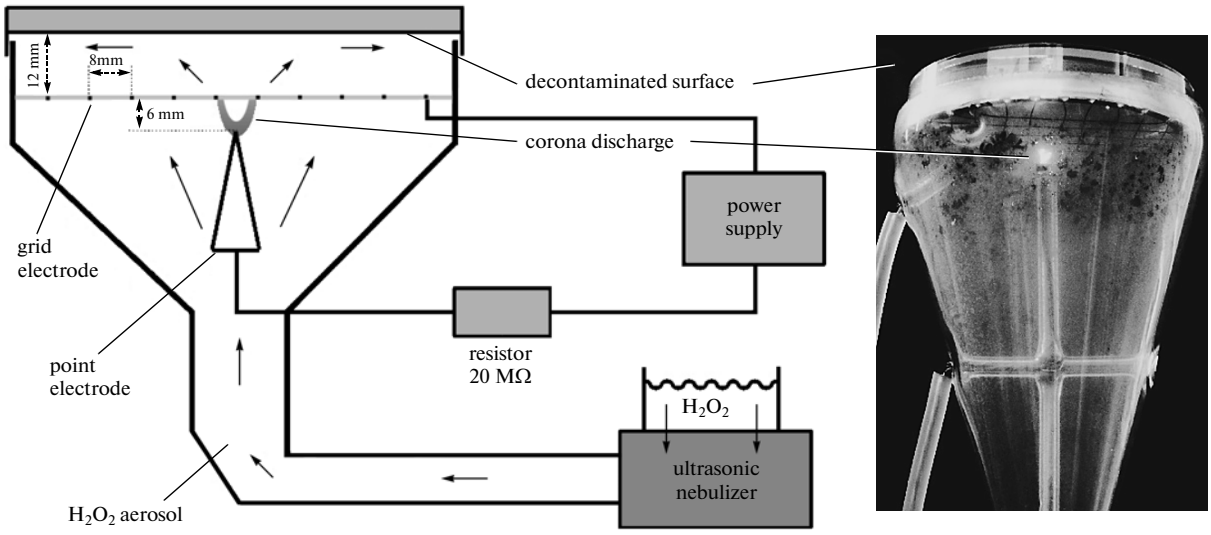

Fig. 1. Schematic experimental arrangement and the picture of the apparatus.

charge stabilisation and its characteristic see e.g. papers [11, 12]. The aerosol of hydrogen peroxide was generated by the ultrasonic nebulizer (Sun-up S.A. Model 3019) and driven into the discharge. The distance between the surface applied on and the grid electrode was set to 12 mm. The schematic experimental arrangement and the picture of the apparatus are shown in Fig. 1.

In experiments the spores were prepared by common microbiological method by taking the loopful of spores from the surface of grown culture and diluting in sterile physiological saline to obtain appropriate concentration. The number of spores in the suspension significantly exceeds the number of other mycelium cells. This initial suspension was diluted to obtain the concentration of approximately 100 cfu/plate or 100 cfu/cm^2 for the experiments with low or high concentration, respectively. The clear YGC agar plates (9 cm in diameter) were used for inoculation. Consequently, the plates were exposed to the solely discharge, solely peroxide aerosol or the discharge burning in the atmosphere of hydrogen peroxide aerosol. After the exposition the exposed plates were incubated at 25°C for 5 days. After this time the survival spores begin to grow and form colonies, the inhibition effect was observed as clear inhibition zones bounded by colonies grown from surviving spores. Due to the size of particular colony the error of the inhibition zone diameter was estimated as 5 mm.

The parameters of discharge and peroxide aerosol varied to test the influence of the discharge polarity and several peroxide concentration. In case of solely discharge, the positive corona discharge burns at the voltage of 3.5 kV, the negative corona discharge burns at the voltage of 2.7 kV. Both voltages correspond to the current of 500 μA. In case of the combination of discharge with the aerosol, the higher atmosphere humidity influences the discharge and to obtain the some current of 500 μA, the voltage was decreased to 3.3 kV and 2.6 kV for the positive and negative discharge, respectively. The influence of the hydrogen peroxide concentration in the aerosol was negligible. Concentrations of hydrogen peroxide water solutions were 0, 3, 10 and 30%. Nebulized hydrogen peroxide was mixed with the air and this aerosol flowed to the discharge area. The flow of the aerosol was adjusted to the values of 2.0 ± 0.1 L/min and the volume concentration of nebulized hydrogen peroxide solution in the mixture was 0.40 ± 0.03 mL/L.

In the first experiment, the characteristic of the spores inactivation of one fungal species Talaromyces striatus was measured for both discharge polarities, various hydrogen peroxide concentration and low spores concentration of 100 cfu/plate. The relatively low concentration was used due to assumption that for some parameters the inactivation may have no or low effect only and in the case of high concentration it may not be visible. T. striatus was selected as known thermoresistant species and frequent contaminant in food processing. Used wild strain also origin as a contaminant from food factory. In the second experiment, due to the results of first experiment one discharge polarity and one peroxide concentration were chosen and the characteristic of spores inactivation was extended to other common fungal species. In this case the inactivation was relatively effective so the high spores concentration of 100 cfu/cm^2 was used to approve the inactivation effect in the conditions of high contamination.

RESULTS AND DISCUSSION

In the first experiment with low concentration of inoculated spores of 100 cfu/plate, the characteristic

of the inactivation of Talaromyces striatus spores for both discharge polarities, various hydrogen peroxide concentrations and exposition times was studied. Results for solely discharge and solely hydrogen peroxide aerosol are shown in Tables 1 and 2, respectively. It is visible that the solely positive corona discharge has no inhibition effect neither for low spores concentration. Contrary to the positive corona discharge, the solely negative corona discharge or solely peroxide aerosol has the microbicidal effect on spores. Results for the combination of hydrogen peroxide aerosol with negative and positive discharges are shown in Tables 3 and 4, respectively and it is visible that the combination of the discharge and peroxide aerosol has synergy effects for the microbicidal efficiency. The combination of the hydrogen peroxide aerosol with the negative corona has higher efficiency than the combination with the positive one. For better visibility, the graph comparing the inhibition zones diameter on the exposition time for solely negative corona discharge, solely aerosol and their combination is shown in the Fig. 2.

Second experiment was focused to the mutual comparison of the inactivation effect on several micromycetes species and has the aim to approve the inactivation in high spores concentration of 100 cfu/cm^2. The apparatus was set to negative corona discharge (2.6 kV, 500 μA) and hydrogen peroxide concentration of 10%, because in this concentration peroxide does not present so high oxidative reagent and in the comparison with 30% concentration the difference in inactivation efficiency is relatively small. The effect was studied on following micromycetes species representing common food contaminants:

1. Aspergillus oryzae (DBM 4002),
2. Cladosporium sphaerospermum (DBM 4282),
3. Alternaria sp. (DBM 4004),
4. Byssochlamys nivea (DBM 4282),
5. Penicillium corylophilum (wild strain),
6. Eurotium amstelodami (wild strain),
7. Talaromyces striatus (wild strain).

The inhibition effect appears as the inhibition zones, the dependencies of the inhibition zones diameter on the time of exposition are shown in the Table 5. Examples of the pictures of agar plates with inhibition zones for Eurotium amstelodami is shown in Fig. 3. In the comparison with low spores concentration the inhibition zones for Talaromyces striatus in high spores concentration have small diameters for 5 s and 20 s of exposition and the much bigger diameters for 60 s and 180 s of exposition. This may indicate that the inactivation effect depends on the concentration of spores weakly (until the spores do not form clusters).

From the first experiment, it may be concluded that the best combination for the inactivation of micromycetes spores is the combination of hydrogen peroxide aerosol and the negative corona discharge. This

Table 1. The dependence of inhibition zones diameter on the exposition time and the discharge polarity for solely discharge effect measured on plates inoculated with low concentration of *Talaromyces striatus* spores (100 cfu/plate)

Dischage polarity	Exposition times [s]	Inhibition zones diameter [mm]
negative (2.6 kV, 500 μA)	5/20/60/180	15/20/30/50
positive (3.3 kV, 500 μA)	5/20/60/180	no inhibition

Table 2. The dependence of inhibition zones diameter on the exposition time for solely peroxide aerosol effect measured on plates inoculated with low concentration of *Talaromyces striatus* spores (100 cfu/plate)

Hydrogen peroxide concentration	Exposition times [s]	Inhibition zones diameter [mm]
3%	5/20/60/180	0/5/8/12
10%	5/20/60/180	0/8/18/40
30%	5/20/60/180	0/12/45/60

Table 3. The dependence of inhibition zones diameter on the exposition time for combined effect of peroxide aerosol with negative corona (2.6 kV, 500 μA) measured on plates inoculated with low concentration of *Talaromyces striatus* spores (100 cfu/plate)

Hydrogen peroxide concentration	Exposition times [s]	Inhibition zones diameter [mm]
0%	5/20/60/180	No inhibition
3%	5/20/60/180	0/10/20/40
10%	5/20/60/180	30/40/80/full
30%	5/20/60/180	35/50/full/full

Table 4. The dependence of inhibition zones diameter on the exposition time for combined effect of peroxide aerosol with positive corona (3.3 kV, 500 μA) measured on plates inoculated with low concentration of *Talaromyces striatus* spores (100 cfu/plate)

Hydrogen peroxide concentration	Exposition times [s]	Inhibition zones diameter [mm]
0%	5/20/60/180	No inhibition
3%	5/20/60/180	0/5/8/12
10%	5/20/60/180	0/8/18/40
30%	5/20/60/180	0/12/45/60

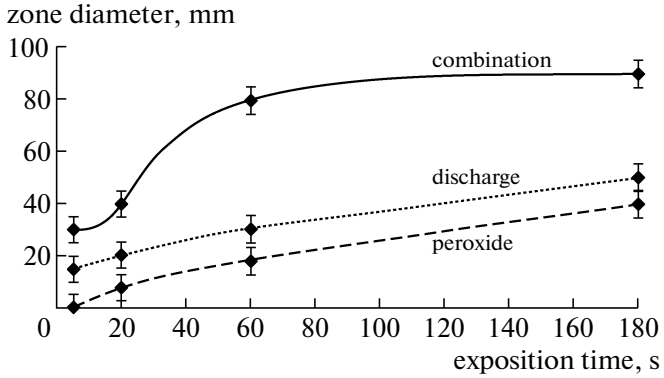

Fig. 2. The dependencies of inhibition zones diameter on the exposition time for solely negative corona discharge, solely hydrogen peroxide aerosol and those combination.

combination is more effective than the use of hydrogen peroxide or negative corona discharge solely.

The second experiment confirmed the inhibition effect for various micromycetes species. The total inhibition was observed for five species only, in the case of *Aspergillus* and *Alternaria* the diameter of inhibition zones increased only to 30 mm. Interesting fact is, that in our experiments *Byssochlamys* and Talaromyces belong to the low resistant ones, but generally they are known as thermoresistant species. At this time it is unable to generalize the differences in resistance between particular species.

The concept of possible mechanisms of synergy effect of peroxide and discharge may be the dissociation of peroxide by electrons produced in the discharge [13]:

$$e^- + H_2O_2 \rightarrow OH^{\bullet} + OH^-.$$

Produced hydroxide radical $OH^{\bullet}$ and hydroxide ion OH^- may cause damage of organic molecules R more efficiently as hydrogen peroxide by the mechanisms proposed in [14, 15]:

$$OH^{\bullet} + RH \rightarrow R^{\bullet} + H_2O,$$
$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet},$$
$$ROO^{\bullet} + e^- \rightarrow ROO^-,$$
$$ROO^- + H^+ \rightarrow ROOH.$$

In addition, we can suggest the application of the Fenton mechanism catalysing the dissociation of hydrogene peroxide, e.g. on the metal electrode:

$$Fe_2^+ + H_2O_2 \rightarrow Fe_3^+ + OH^{\bullet} + OH^-,$$
$$Fe_3^+ + H_2O_2 \rightarrow Fe_2^+ + OOH^{\bullet} + H^+.$$

Produced peroxide radical $OOH^{\bullet}$ commonly dissociates to produce superoxide radical $O_2^{-\bullet}$ (which may living cells convert back to hydrogen peroxide by the enzyme superoxide dismutase):

$$OOH^{\bullet} \rightarrow O_2^{-\bullet} + H^+.$$

However, in discharge it may be dissociated to produce hydroxide radical and hydroxide ion [13, 16]:

$$e^- + OOH^{\bullet} \rightarrow OH^{\bullet} + O^-,$$
$$e^- + OOH^{\bullet} \rightarrow OOH^-,$$
$$e^- + OOH^- \rightarrow OH^- + O^-.$$

In addition, the fact that the combination of hydrogen peroxide with negative corona discharge exhibits better inactivation effect as with the positive one may be explained by higher presence of electrons (or negative

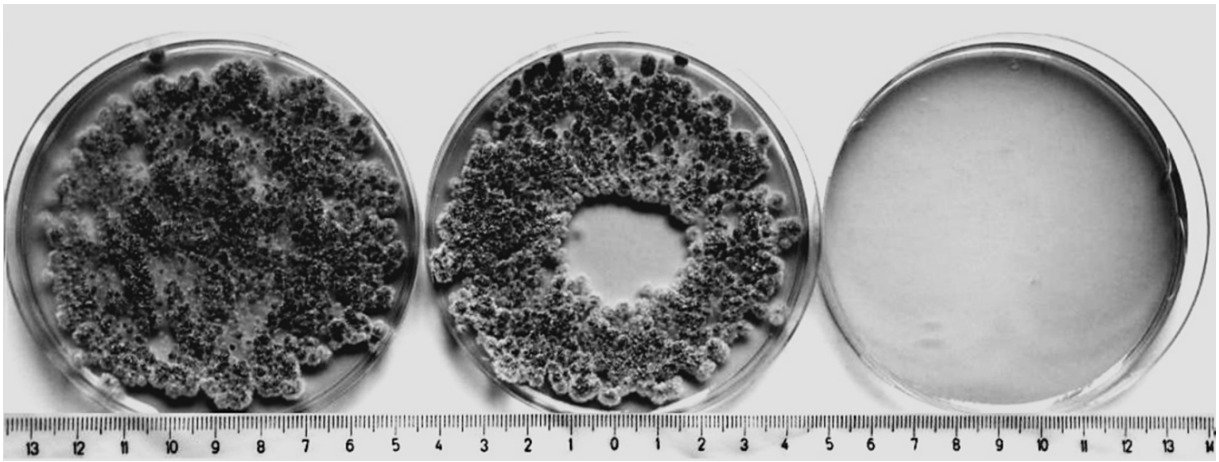

Fig. 3. Growth inhibition of *Eurotium amstelodami* by the combined treatment with the negative discharge and 10% hydrogen peroxide aerosol; control (left), exposed for 20 s (middle) and 180 s (right).

Table 5. Inhibition effect of negative corona discharge with the combination of 10% H_2O_2 aerosol measured on plates inoculated with high concentration of spores (100 cfu/cm^2)

Micromycete species	Exposition times [s]	Inhibition zones diameters [mm]
Aspergillus oryzae	5/20/60/180	5/15/15/30
Cladosporium sphaerospermum	5/20/60/180	5/5/60/full
Alternaria sp.	5/20/60/180	0/0/30/30
Byssochlamys nivea	5/20/60/180	10/10/30/full
Penicillium corylophilum	5/20/60/180	5/15/40/full
Eurotium amstelodami	5/20/60/180	5/25/full/full
Talaromyces striatus	5/20/60/180	20/35/80/full

ions) in negative discharge and following more efficient dissociation of hydrogen peroxide.

CONCLUSION

The inhibition effect of hydrogen peroxide aerosol, positive and negative corona discharge and their combinations has been studied on the spores of *Talaromyces striatus*. The solely positive corona discharge exhibits no inactivation effect. The solely negative corona discharge and solely hydrogen peroxide aerosol exhibit the inactivation effect, but their combinations exhibit to be much more effective. This inactivation effect was confirmed on seven different micromycetes species and the possible mechanisms of the hydrogen peroxide and corona discharge reciprocal synergy action was proposed.

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