Research Article

Inactivation of Microbial Food Contamination of Plastic Cups Using Nonthermal Plasma and Hydrogen Peroxide

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The inactivation effect of the combination of nonthermal plasma and hydrogen peroxide aerosol for the microbial decontamination of inner surface of cylindrical container buckets is studied on one bacterial and seven filamentous micromycete species and on airborne-contaminated cups. While the decontamination by single nonthermal plasma or hydrogen peroxide is not observable after 120 s, the strong decontamination by their combination occurs after 30 s of exposure. Moreover, observed total elimination of airborne contamination of plastic cups predetermines this method as a suitable alternative to the currently used method based on the application of hydrogen peroxide.

1. Introduction

The microbial contaminants present a serious problem, among others, in food processing, and their elimination is often desired in many areas. There are many papers studying the microbial decontamination of food and food packaging materials by conventional as well as alternative physical or chemical methods, for example, reviews [1, 2] or research articles as [3, 4] comprise the ultrahigh pressure processing (cold pasteurization), ionizing radiation, electron beam, UV radiation, pulsed electric field, magnetic field, ozone, or other chemical agents as peracetic acid, acetic acid, hydrogen peroxide, linalool, carvacrol, or thymol. Ultrasound is used in [5], pulsed light systems are used in [6] or [7], the effect of radio frequency heating is used in [8], and recently also the shaped electrical pulses have been studied [9].

One possible alternative presents the microbial decontamination by nonthermal plasma (NTP). There are already a lot of works describing its biological effects, devoted mainly to the killing of bacteria or various applications in medicine. See, for example, reviews [10, 11] or other studies [12–18]. The contamination may often arise also from the packaging materials [19, 20]; therefore, many papers describe basic studies of NTP application in food packaging. Several papers deal with decontamination of food packaging materials by NTP generated under low pressure as [21–25]. Following works use NTP in more economically and easier-to-use form at atmospheric pressure, for example, the decontamination of polymer foils [26], plastic tray, aluminum foil and paper cup [27], PET film [28], dried laver [29], shell eggs [30], or sealed packages [31]. Also other effects of NTP treatment on food packaging materials as

FIGURE 1: Schematic view of the apparatus and picture of plasma active area. Nebulized hydrogen peroxide mixed with air flows to the discharge active plasma region where active particles are produced and carried by the ion wind to the sample.

influence on contact angle, wettability, roughness, and surface energy are studied and summarized in [32, 33].

To increase the decontamination efficiency, the atmosphere where the plasma is generated may be also enriched by some other microbicidal substances, for example, hydrogen peroxide or peracetic acid. Such commercial sterilizers already exist; however, they work at reduced pressure and massive apparatus, resembling autoclave, is needed; for details, see, for example, the review [11] or [34].

In this paper, we combine the benefits of NTP generated at atmospheric pressure, where no additional vacuum apparatus is necessary, and the addition of other bactericidal agent—hydrogen peroxide. This paper follows our previous articles [35, 36], where synergy decontaminating effect of the combination of NTP with hydrogen peroxide aerosol was demonstrated on agar surfaces and dry cellophane foils. Here, we present the study of volume decontamination effect of plastic objects on both defined and airborne contamination. As first, we confirm the previously observed synergy effect in volume also. Following, we have studied the decontamination of large volumes with high concentration of microorganisms so that we could subsequently demonstrate the reliable inactivation of much lower airborne contamination of packaging vessels.

2. Materials and Methods

2.1. Plasma Generation. The NTP was generated in the apparatus previously described in [35, 36]. The point-to-wire discharge burns between the point and grid electrode in an open chamber where the hydrogen peroxide or pure water aerosol was added to the air atmosphere. The point electrode

was represented by the tip of a syringe needle Medoject (0.6×30 mm) situated vertically to the grid electrode. The grid consisted of stainless steel wire of 0.25 mm diameter forming the net with a mesh size of 8 mm. The electrode distance was adjusted to 6 mm. The discharge was stabilized by the connection of a serial resistance of 10 MΩ into the circuit. The polarity of the point electrode was set as negative and the grid electrode as positive. The discharge voltage was set to 2.7 kV which corresponds to the current of 500 μA. For more details about this discharge denominated as pulseless glow, see our previous paper [37], where several stabilized discharges are studied. For other information about the corona discharge stabilization and its characteristic, see, for example, papers [38, 39]. The aerosol of pure water or 10% hydrogen peroxide was generated by the ultrasonic nebulizer (Lucky Reptile, Super Fog SF-1) and mixed with the air; the volume of nebulized water or hydrogen peroxide in the air mixture was 0.40 ± 0.03 ml/l (determined by the weight loss). This mixture flowed to the discharge area and created the discharge atmosphere. The aerosol flow was adjusted to the value of 2.0 ± 0.1 l/min (determined by ebulliometry). The distance between the decontaminated object and the grid electrode was set to 12 mm. The schematic experimental arrangement and the picture of plasma active area are shown in Figure 1.

2.2. Microorganisms under Study. The microorganisms under study were bacteria *Staphylococcus epidermidis* (wild strain) and filamentous micromycetes *Aspergillus oryzae* (DBM 4002), *Aspergillus niger* (DBM 4054), *Cladosporium sphaerospermum* (DBM 4282), *Alternaria* sp. (DBM 4004), *Eurotium* sp. (wild strain 1), *Eurotium* sp. (wild strain 2), and *Trichoderma atroviride* (wild strain). DBM is the Collection of Yeasts and Industrial Microorganisms of the Department of Biochemistry and Microbiology at the University of Chemistry and Technology, Prague. Wild strains were contaminants isolated from food products. In all cases, the bacteria were cultivated on the Mueller-Hinton agar (Oxoid) at 37°C overnight for 18 hours and the micromycetes were cultivated on the Sabouraud medium (Oxoid) agar at 25°C for 5 days to become sporulated.

The suspension of each microorganism under study was prepared by the mixing of loopful taken from the surface of grown culture into the sterile water; in the case of micromycetes, Tween 80 in ratio 0.1% was added into the suspension to improve wettability. It was determined by microscopic examination that the number of conidiospores in the suspension significantly exceeds the number of other mycelium cells.

2.3. Decontamination of Container Buckets. The volume decontamination effect was examined on cylindrical polytetrafluoroethylene (PTFE) container buckets of heights of 13 cm and diameters of 7 cm and 10 cm for volumes of 0.5 l and 1 l, respectively. The inside of containers was lined with contaminated cellophane foils which simplify the microbial manipulation. Prepared suspension of microorganisms was spread homogeneously onto the surface of cellophane foil in

FIGURE 2: Systematics of the experiment. Cellophane foil is inoculated by the microbial suspension, dried, cut into the strips, and placed on the inner surface of cylindrical containers. Containers are exposed to the NTP with hydrogen peroxide aerosol, strips are imprinted onto the surface of the agar medium and cultivated, and the inhibition zones are determined.

the concentrations of 10^4 cfu/cm² and 10^3 cfu/cm² for bacteria and micromycetes, respectively. After one hour drying at common room temperature (22°C) and humidity (40%), the foil was cut into strips (2 cm × 33 cm for 0.5 l cup and 2 cm × 36 cm for 1 l cups) and placed on the inner surface of cylindrical containers and exposed to one of described decontamination methods. To transfer the spores from foils to agar, both the exposed and reference foils were imprinted onto the surface of agar and incubated at 37°C for 1 day or at 25°C for 5 days in the case of bacteria or micromycetes, respectively. The operating sequence together with the particular sizes of used foils and cylinders is depicted in Figure 2. All experiments were performed in triplicate.

In first set of exposures, our previous study 36 was followed to confirm the inactivation properties of NTP for 3D objects. Moreover, also the spatial distribution of inhibition effect efficiency was determined. The prepared samples of 0.5 l containers with limited number or micro-organism species *S. epidermidis*, *C. sphaerospermum*, and *A. niger* were exposed to NTP with addition of pure water aerosol, to NTP with addition of hydrogen peroxide aerosol, and to the hydrogen peroxide aerosol without the NTP. Samples were exposed for 5, 10, 15, 30, 60, and 120 s. The inhibition effect was denoted as full for no colony growth, as partial for observable isolated colonies (lower than approx. 30 cfu/cm²), and the continuous overgrown (higher than approx. 30 cfu/cm²) was denoted as no inhibition.

Afterwards, the efficiency of inactivation for a broader spectrum of all mentioned microorganisms was examined in both container types. Prepared samples were exposed for 30, 60, and 120 s to the combination of NTP with hydrogen peroxide aerosol. The inhibition effect was observed both as the inhibition zone without any growth of colony and as the number of grown cfu.

2.4. Decontamination of Commercial Cups. To confirm the decontamination effect of used apparatus for direct practical application, the decontamination was studied on commercial polypropylene (PP) 0.5 l cups (Wimex s.r. o., Czech Republic) of height of 13.5 cm and of bottom and top diameter of 5.5 cm and 9 cm, respectively. Twenty clean cups were contaminated by artificial or airborne contamination.

For artificial contamination, the inside surface of a cup was sprayed homogeneously by 2 ml of water suspension of *A. oryzae* or *Alternaria* sp. in concentration of 10^3 cfu/ml with Tween 80 in ratio 0.1% and dried for one hour at common room temperature (22°C) and humidity (40%). Consequently, cups were exposed for 10, 15, 30, 60, and 120 s to the combination of NTP with hydrogen peroxide aerosol.

For airborne contamination, cups were kept open in the laboratory for one week and ten of them were exposed to the NTP with hydrogen peroxide aerosol for 30 s.

TABLE 1: Decontamination effect of all used methods in 0.5l cylindrical containers for *S. epidermidis*, *C. sphaerospermum*, and *A. niger* indicated only qualitatively.

Method	Microorganism	Extent of decontamination after exposure time (s)						
		0	5	10	15	30	60	120
NTP with hydrogen peroxide aerosol	<i>S. epidermidis</i>	No	Full	Full	Full	Full	Full	Full
	<i>A. niger</i>	No	No	Partial	Full	Full	Full	Full
	<i>C. sphaerospermum</i>	No	No	Partial	Partial	Full	Full	Full
NTP with pure water aerosol	<i>S. epidermidis</i>	No	No	No	No	No	No	No
	<i>A. niger</i>	No	No	No	No	No	No	No
	<i>C. sphaerospermum</i>	No	No	No	No	No	No	No
Hydrogen peroxide aerosol	<i>S. epidermidis</i>	No	No	No	No	No	No	No
	<i>A. niger</i>	No	No	No	No	No	No	No
	<i>C. sphaerospermum</i>	No	No	No	No	No	No	No

FIGURE 3: The spatial distribution of inhibition effect for *C. sphaerospermum* for several exposure times.

After, all cups were filled with prepared potato-carrot broth with the addition of 50 g of glucose per 1l as model content, closed to prevent contamination, and kept in room temperature for one week. Finally, the number of grown micromycetal colonies on the broth surface was counted. All experiments were performed in triplicate.

3. Results and Discussion

Table 1 shows the results for decontamination of samples contaminated by *S. epidermidis*, *C. sphaerospermum*, and *A. niger* in 0.5l cylindrical container buckets by all three mentioned methods. Obtained results were identical for all three particular repetitions. Observable decontamination occurred after the exposure to the NTP with hydrogen peroxide aerosol only, where the full decontamination occurred within 5 s of exposure for *S. epidermidis* (4 log₁₀ reduction) and within 30 s of exposure for *C. sphaerospermum* and *A. niger* (3 log₁₀ reduction). For other methods, the decontamination was not observable, so that it cannot be declared neither as inhibition zone nor countable colonies even after 120 s. These results correspond with our previous observation and confirm that the combination of NTP and hydrogen peroxide aerosol is much more efficient than their single action for volume decontamination also.

On this basis, in following experiments, the samples were exposed to the NTP in the aerosol of hydrogen peroxide only. The spatial distribution of inhibition effect for *C. sphaerospermum* is depicted in Figure 3. The inhibition

TABLE 2: Decontamination effect of NTP with hydrogen peroxide aerosol in 0.5l cylindrical containers indicated as both inhibition zone (% of area) and total cfu for exposure time (*t*).

Microorganism	Inhibition zone (% of area)/grown cfu		
	<i>t</i> = 30 s	<i>t</i> = 60 s	<i>t</i> = 120 s
<i>S. epidermidis</i>	100/0	100/0	100/0
<i>A. niger</i>	100/0	100/0	100/0
<i>C. sphaerospermum</i>	100/0	100/0	100/0
<i>Eurotium</i> sp. strain 1	100/0	100/0	100/0
<i>Eurotium</i> sp. strain 2	100/0	100/0	100/0
<i>A. oryzae</i>	70 ± 5/10 ± 5	100/0	100/0
<i>T. atroviride</i>	75 ± 5/10 ± 5	100/0	100/0
<i>Alternaria</i> sp.	60 ± 5/30 ± 5	80 ± 5/5 ± 3	100/0

TABLE 3: Decontamination effect of NTP with hydrogen peroxide aerosol in 1l cylindrical containers indicated as both inhibition zone (% of area) and total cfu for exposure time (*t*).

Microorganism	Inhibition zone (% of area)/grown cfu		
	<i>t</i> = 30 s	<i>t</i> = 60 s	<i>t</i> = 120 s
<i>S. epidermidis</i>	100/0	100/0	100/0
<i>A. niger</i>	100/0	100/0	100/0
<i>C. sphaerospermum</i>	100/0	100/0	100/0
<i>Eurotium</i> sp. strain 1	40 ± 5/30 ± 5	100/0	100/0
<i>Eurotium</i> sp. strain 2	35 ± 5/100 ± 10	100/0	100/0
<i>A. oryzae</i>	30 ± 5/10 ± 5	70 ± 5/5 ± 3	100/0
<i>T. atroviride</i>	20 ± 5/10 ± 5	80 ± 5/5 ± 3	100/0
<i>Alternaria</i> sp.	35 ± 5/uncount	75 ± 5/20 ± 5	80 ± 5/5 ± 3

zone becomes to be visible after 10 s exposure in the center which corresponds to the bottom of buckets, and for longer exposure times, it enlarges and takes the whole area. The gradual extending of inhibition zone from the center corresponds with the airflow induced by the discharge ion wind, which is a characteristic of point-to-point/wire/plane discharges. For more details about the plasma sources and their properties, see, for example, our previous works [40, 41] dealing with point-to-point cometary discharge, demonstrative work about the ion source induced airflow [42], or the review of several common plasma sources [43] suitable for decontamination.

TABLE 4: Decontamination effect of all used methods in 0.5l cylindrical containers for *A. oryzae* and *Alternaria* sp.

Microorganism	Grown cfu					
	$t = 0$ s	$t = 10$ s	$t = 15$ s	$t = 30$ s	$t = 60$ s	$t = 120$ s
<i>A. oryzae</i>	Uncount	5	0	0	0	0
<i>Alternaria</i> sp.	Uncount	Uncount	500 ± 100	50 ± 10	4 ± 3	0

FIGURE 4: The picture of exposed (upper ten) and nonexposed (bottom ten) airborne-contaminated cups after cultivation.

The results of decontamination of all species for both sizes of containers are presented in Tables 2 and 3. In 0.5l containers, the full decontamination occurs for most of species after shortest exposure of 30 s and the full decontamination for all species occurs after exposure of 120 s. In 1l containers, the decontamination after 30 s was only partial for most of species and the total decontamination occurred after 120 s of exposure for all species except *Alternaria* sp. The results show that the bacterium *S. epidermidis* is more sensitive than all micromycetes species and that from selected micromycetal species, *Alternaria* sp. is the most resistant one. As expected, the longer exposure times are necessary for larger volume buckets.

The results for decontamination of artificially contaminated commercial PP cups are given in Table 4. The number of cultivable cfu decreases with the time of exposure. For *A. oryzae*, representing the sensitive species, the strong inhibition occurs after 10 s of exposure and the full inhibition occurs for 15 s and longer exposures. For the most resistant one, *Alternaria* sp., the inhibition effect is visible after 15 s of exposure and the full inhibition occurs after 120 s exposure. For the airborne contamination, it was found that for nonexposed cups, approximately three grown micromycete colonies per one cup are observed (2.5 ± 2.2); however, in exposed cups, no grown colonies are observed. These results make this synergy effect of hydrogen peroxide and nonthermal plasma potentially applicable to the practice. For example, the jam or ketchup production companies in Czechia have permanent problems with spoilage in plastic-packed products. The picture of cups after cultivation is shown in Figure 4. In this case, the number of cfu does not represent the real number of culturable cfu in cups, but it has to demonstrate the potential of present microorganisms to spoil the content, Q.E.D.

In our previously mentioned work [37], the discharge denominated as pulseless glow almost identical with that one used in this study. The discharge burned under similar geometry and identical electrical and chemical characteristics: in time constant voltage of 2.7 kV and current of $500 \mu\text{A}$ where no pulses were recorded by 150 MHz bandwidth oscilloscope; emission spectrum was identical with pulseless glow one. The dominant nitrogen N_2^+ bands were detected; the lower intensity of N_2^+ indicates the high electron temperature important for microbicidal activity [44, 45]; also the minute peak attributed to $\text{OH}^\cdot$ was detected. Fact that the presence of hydrogen peroxide aerosol in the discharge atmosphere did not change its evaluated characteristics indicates that the rapid increase of microbicidal activity must be induced in the postdischarge dark phase. The main mechanisms of hydrogen peroxide activity may be its dissociation (for details, see our previous paper [35]):

Presented apparatus seems to be a potential alternative to several common decontamination methods based on hydrogen peroxide. Addition of the NTP part to current peroxide-based methods would be very easy and cheap and may help to increase the decontamination efficiency, increase the speed of production line, or decrease the amount or concentration of applied peroxide, or solve the contamination problems in production lines where it is difficult to ensure fully aseptic conditions. The decrease of concentration of hydrogen peroxide or amount of energy is also important from an ecological point of view.

4. Conclusions

Experimental study of the volume decontamination effects of nonthermal plasma generated in corona discharge in combination with hydrogen peroxide aerosol shows much higher efficiency than the single action of NTP or hydrogen peroxide. While the decontamination of single nonthermal plasma or hydrogen peroxide is not observable after 120 s, the strong decontamination effect of their combination occurs after 30 s of exposure. This phenomenon was demonstrated on one bacterial and seven micromycete species in both container buckets and 0.5l commercial cups. Along with the total inactivation of airborne contamination of commercial cups, it predetermines this method as a suitable alternative to the currently used methods based on hydrogen peroxide applications. It may help to reduce the required amount or concentration of hydrogen peroxide, which is also important from an ecological point of view.

Data Availability

In Tables 1–4, only the mean values and standard deviations are presented. The reader can access the particular results on request to the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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