https://doi.org/10.46813/2023-146-151

STUDY OF THE FUNGICIDAL PROPERTIES OF OZONE TREATMENT, Ag AND Cu NANOPARTICLES AND THEIR COMBINED ACTION ON THE MODEL OF SANITARY SIGNIFICANT MOLD SAPROPHYTE *ASPERGILLUS FLAVUS*

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A study was conducted to determine the fungicidal activity of a sanitary contaminant of feeds – representatives of the genus *Aspergillus Mich.* – species of Aspergillus flavus using the ozone treatment method, treatment with the composition of Ag and Cu nanoparticles and the combined effect of these methods. When studying the combined effect of ozone, NPAg and NPCu, it was found that at the concentration ratio of 100:100 μg/cm^3, there was a fungicidal effect on museum strains of *Aspergillus flavus* test cultures, i.e., the combination with ozone enhanced the antifungal properties of nanocomplex.

PACS: 52.80. Hc, 52.90.+z,52.75.−d

INTRODUCTION

A comprehensive, safe and responsible approach to the quality of livestock products and the country's food policy is the basis of EU requirements. Abroad, the problems of quality and safety for food products of animal origin are of major importance. Such studies are conducted in the USA (FDA), EU and in a number of international organizations (WHO, FAO, etc.).

It is known that the health and productivity of farm animals depend not only on the quantity and quality of feed. In today's conditions of intense climate changes, violation of technologies for obtaining agricultural products and uncontrolled use of a large number of feed additives and low-quality raw materials for their production causes an increase in the content of toxic substances in the organisms of animals. This has a negative impact on their development and productivity. Also, there is a risk of metabolites of toxic substances getting into the products. It is possible to change some physical and chemical indicators, in particular, the activity of certain enzymes, insufficient content of trace elements and vitamins, which significantly affects the quality and safety of products, in particular milk, meat and eggs [1].

The presence of biotic contaminants – micromycetes – is one of the main factors characterizing the degree of safety for feed raw materials. In accordance with FAO, from 25 to 30% of grain products produced in the world are contaminated with micromycetes [1]. There is an extremely wide area of distribution for microscopic fungi. Unfavorable climatic conditions and violations of storage technology result in the accumulation of saprophytic and pathogenic micromycetes (*Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus amstelodami, Penicillium brevicaulis, Penicillium bicolor, Fusarium, Mucor, Rhizopus*), changes in the composition, taste and nutritional value of feed. As a result, the ingestion of food infected with pathogenic micromycetes can cause mycosis and mycotoxicosis [2].

The number and species composition of mold fungi, which are currently identified during mycological studies of feed, are constantly growing. Therefore, researchers are constantly looking for the ways to resolve the issue of their timely detection and development of decontamination methods. Scientific support in this direction is absolutely necessary and serves as a guarantee of national security. Prevention of possible losses from feed contaminated with mycobiota is necessary for further long-term social prediction and assessment of the effectiveness of environmental protection measures aimed at preventing environmental pollution [3, 4].

Due to its high oxidation potential, ozone interacts with many organic substances in the protoplasm of bacterial cells and destroys them. The biocidal effect of ozone is a result of its reaction with fatty acids in the cell walls and membranes of bacteria, in the protein shells of viruses. In the case of bacteria, oxidation leads to the changes in the permeability of cell walls and the transition of intracellular fluid into solution. For the viruses, a change in the protein shell prevents their capture by susceptible cells.

Ozone has a strong bactericidal, virulicidal, sporicidal and fungicidal effect. In particular, the highest sensitivity to ozone was observed both for test bacteria and pathogenic ones. The parameters of effective treatment modes depend on water quality, technology being used and design of the facility at the specific objects [5, 6].

However, despite all the advantages, the ozone treatment method has a number of disadvantages. Ozone is not sufficiently active in the destruction of phenolic compounds. During ozone treatment, carcinogenic formaldehyde is formed. Ozone belongs to a high class of dangerous substances, so its use is complicated by the need to comply with the safety rules [7].

The search for new methods of water purification that would eliminate the disadvantages of ozone treatment is an urgent task, in particular, amidst the COVID-19 pandemic.

The development of disinfection technology using nanotechnology is of great interest to both representatives of scientific community and potential feed producers and consumers. The size of nanoparticles from 1 to 100 nm allows their penetration into various biological objects, including spores and mycelium of microscopic fungi [8 - 10]. In particular, it is known that a high fungicidal effect

is found for Ag nanoparticles with the size of about 3 nm in relation to *the Candida pathogenic species and Trichophyton mentagroptytes.* For colloidal *Ag* at the concentration of 1…13 μg/ml, 80% inhibitory concentration (IC80) is observed [8], and 100% inhibition is obtained for phytopathogenic fungi when using silica-silver nanoparticles at the concentration of 10 μg/ml [9].

Based on the results of previous studies, a series of experiments was conducted, during which fungistatic properties of Ag and Cu nanoparticles were observed at the concentrations of 150 μg/cm^3 after the exposure period of 60 and 180 min. In particular, the average number of colonies grown for the *Aspergillus flavus* test culture was 16.8 and 3.8; 39.7 and 35.2. Dispersions of Fe nanoparticles and *Mn* dioxide in all the experimental concentrations did not show either fungicidal or fungistatic properties. Continuous growth of *Aspergillus flavus* was observed for all dilutions [11].

When studying fungicidal (fungistatic) properties of ozone treatment for an aqueous solution contaminated with *Aspergillus flavus* test culture, ozone demonstrated its fungicidal properties against this test culture (95…98% growth inhibition) [12].

High fungicidal properties are demonstrated both for ozone treatment and nanotechnology. There are reasons to believe that the symbiosis of these two methods allow increasing the efficiency of water and feed decontamination due to their synergistic effect.

MATERIALS AND METHODS

Determination of the fungicidal activity for *Ag* and *Cu* nanoparticle compositions in the concentration range was carried out using the methods of mycological analysis generally accepted [13] and developed by the NSC "Institute of Experimental and Clinical Veterinary Medicine" [14] on the model of a sanitary significant feed contaminant – a representative of the genus *Aspergillus Mich* [15] of the museum strain *Aspergillus flavus.*

Experimental samples of metal nanoparticles were synthesized using the chemical condensation method by restoring the corresponding metal salts in an aqueous solution and standardized in accordance with their stability and size at the Biocolloidal Chemistry Institute named after F.D. Ovcharenko of NASU. The average size of the experimental samples for metal nanoparticles was calculated using the method of laser correlation spectrometry. A laser correlation spectrometer Zetasizer-3 ("Malvern Instruments Ltd", Great Britain) was used to determine the diffusion rate constant and hydrodynamic diameter of the particles (Rawle A., www.malvern.co.uk). Thus, the experimental samples of the following metal nanoparticles were used in the study: Ag (initial concentration – 86.4 μg/cm^3 by metal, average size (~31.5±0.9) nm) and Cu (initial concentration – 2678.0 μg/cm^3, medium size (~70.0±4.0) nm), respectively.

The concentration range of the experimental samples for metal nanoparticles, which would show fungicidal (fungistatic) properties, was determined in accordance with the previous studies of an aqueous suspension of the *Aspergillus flavus* test culture. The concentration ratio for NPAg and NPCu samples, which would show fungicidal (fungistatic) properties, was determined on colloidal dispersions of nanoparticles. During the experiment, four experimental solutions of nanoparticle compositions (Ag:Cu) were prepared in the ratio (1:1) of the following concentrations: composition I – 100:100 μg/cm^3; composition II – 150:150 μg/cm^3; composition III – 100:150 μg/cm^3 and composition IV – 150:100 μg/cm^3 by metal, respectively. Exposure period at the temperature of (19±1.0)°C was 1, 2, and 3 h.

Aspergillus flavus test culture was standardized by the number of spores in 1.0 cm^3 and the working dilution was 120 spores in 1/5 mm^3. Selection of the strain, verification of its vital activity after storage at the temperature of (6±2)°C was carried out by sowing the test culture in test tubes on a dense nutrient medium (agar wort) after exposure at room temperature for at least one hour.

Determination of the fungicidal properties for ozone and its combined action with nanoparticles of Ag and Cu metals against the most resistant strain of *Aspergillus flavus* was carried out using a specially developed and manufactured experimental stand for ozone treatment of solutions. On the experimental stand for ozone treatment of solutions, ozone synthesis from laboratory air was performed using the barrierless ozone generator. After this, the resulting ozone-air mixture was bubbled in the solution.

The experimental stand (Fig. 1) included the following functional units: air compressor Secoh sangyo (Japan) with the productivity of 50 l/min and maximum pressure up to 12.7 kPa, gas flow meter (PM-4 GU3), laboratory ozone generator "StreamOzone", ozone monitor Teledyne Instruments (USA) model 454H with the measurement range of 0.1…100 g/m^3, laboratory macros for placing samples of the solution being studied and ozone destructor. Ozone concentration in the ozone-air mixture was 10 g/m^3 at the flow rate of 8 l/min.

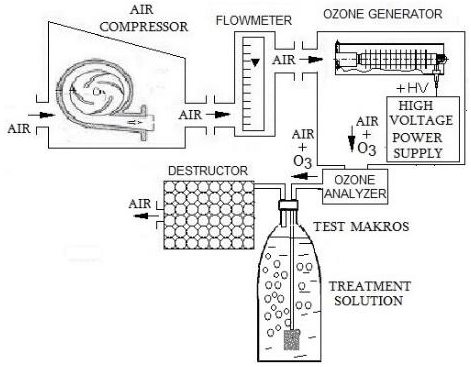

Fig. 1. Ozone treatment system for the aqueous solution under study

In Fig. 2, the process of *Aspergillus flavus* aqueous solution treatment with ozone-air mixture is demonstrated.

Fig. 2. Treatment of Aspergillus flavus solution with ozone-air mixture

During the experiment, fungicidal properties of Ag and Cu solutions, ozone and their combined action were determined by the following scheme: the working aqueous suspension of the *Aspergillus flavus* culture in the amount of 1.0 was added to 9.0 cm^3 of the abovementioned four experimental solutions of nanoparticle compositions (Ag:Cu) in the ratio (1:1). Exposure period at the temperature of (19±1.0)°C was 1, 2. and 3 h.

Control samples were prepared: the "positive" one was sterile tap water and the "negative" one was water with the addition of nystatin (100 cm^3 of the medium – 100.000 units of nystatin). The number of surviving spores was determined by sowing the test samples in Czapek agar cooled to (45±0.5)°C, incubation in a thermostat at the temperature of (26.0±1.0)°C and counting the number of colonies on the third, fifth and seventh day.

Upon the end of cultivation within the specified period, a macroscopic examination of the cultures was carried out (external signs of micromycete colonies, characteristic features of growth, color, shape, consistency of colonies, presence or absence of sclerotia, pigment, etc. were determined) and compared with the museum strain [15].

To calculate the average result on the number of fungal colonies grown during determination of the fungicidal activity for the compositions of metal nanoparticles and their combination with ozone on the model of *Aspergillus flavus* museum strain, the experiments were repeated three times.

A positive fungicidal composition was considered to be the one in which there was a 95…98% death of spores of the test cultures by the results of at least 3 experiments in the presence of spore growth in the positive control sample.

Based on the experimental results obtained at different times, the average number of colonies was calculated.

EXPERIMENTAL RESULTS

In the course of experiments, it was found that ozone treatment of water contaminated with a test culture *Aspergillus flavus* standardized by the number of spores had fungistatic properties with respect to the test culture after exposure for 1 h. Exposure for 2 and 3 hours significantly affected the number of colonies, which amounted to 14 and 0, respectively, in comparison with the continuous growth of *Aspergillus flavus* in the positive control sample. Thus, fungicidal properties of ozone were revealed after 2 and 3 h of exposure.

When determining fungicidal (fungistatic) effect of NPAg and NPCu, it was found that such a treatment affected the growth activity of spores of *Aspergillus flavus* test cuture. In particular, the average number of *Aspergillus flavus* colonies (see Table below) growing after exposure period of 3 h was 60.0 CFU on the 7th day of incubation at the NPAg and NPCu concentration of 100:150 μg/cm^3. At the concentration of 150:100 μg/cm^3, it was 35 CFU. At the concentration of 150:150 μg/cm^3, the test culture growth was not detected for any exposure and registration period compared to the continuous growth of microscopic fungi in the "positive" control sample.

When studying fungicidal (fungistatic) effect of ozone in combination with NPAg and NPCu, it was found that such a treatment revealed significant fungistatic properties. In particular, the average number of *Aspergillus flavus* colonies growing on the 7th day of incubation at the NPAg and NPCu concentration of 100:100 μg/cm^3 for 1 h exposure was 27.0 CFU, for 2 h exposure it was 11.0 CFU and for 3 h exposure, the growth of micromycetes was not found. At the concentration of 150:100 μg/cm^3 and exposure for 2 h, the number of colonies was 49.0 CFU and after exposure for 3 h, it was 29.0 CFU. At the NPAg and NPCu concentration of 150:150 μg/cm^3, the growth of the test culture was not detected for any exposure and registration period compared to the continuous growth of microscopic fungi in the "positive" control sample.

The results of studies on determining fungicidal activity of Ag and Cu nanoparticle compositions and their combination against the growth activity of spores of *Aspergillus flavus* museum strain at the temperature of (18.0±0.5)°C on the 7th day of observation are presented in the Table below.

Results on determining fungicidal (fungistatic) properties of the solution contaminated with Aspergillus flavus on the 7th day of observation

Treatment method		Average number of colonies, CFU (treatment hours)		
		1	2	3
Nanocomplex (Ag:Cu)	100:100	+	+	**103.0**
	150:150	-	-	-
	100:150	**82.0**	**78.0**	**60.0**
	150:100	+	**55.0**	**35.0**
Ozone treatment in combination with NPAg and NPCu	100:100	**27.0**	**11.0**	-
	150:150	-	-	-
	100:150	**67.0**	**34.0**	**28.0**
	150:100	+	**49.0**	**29.0**
"Positive" control sample		+	+	+
"Negative" control sample (with nystatine)		-	-	-

Notes:
1. – no growth of *Aspergillus flavus*;
2. + continuous growth of *Aspergillus flavus;*
3. n/ind. growth – unidentifiable growth of the test culture.

On the basis of the experimental results, the antimycotic Ag and Cu nanocomplex with a fungicidal (fungistatic) effect on museum strains of *Aspergillus flavus* test cultures in the concentration ratio of 150:150 μg/cm^3 (by metal) was selected for the development of water disinfection technological process. To significantly reduce the concentration of Ag and Cu in the disinfection process, a pre-tretment of the solution with ozone-air mixture at the exposure period of 1 hour followed by the use of Ag and Cu nanocomplex with the concentration of 100:100 μg/cm^3 was recommended.

CONCLUSIONS

1. When studying fungicidal (fungistatic) properties of ozone-air treatment (ozone concentration in the

mixture was 10 g/m^3) for the aqueous solution of the museum strain of *Aspergillus flavus* test culture, fungistatic properties were revealed after exposure period of 1 h, and fungicidal properties were revealed after 2 and 3 h of exposure.

2. When determining fungicidal properties of Ag and Cu nanoparticle compositions in relation to the test culture *A. flavus*, it was found that nanoparticles in the concentration ratio of 150:150 μg/cm^3 (by metal) revealed fungicidal properties after exposure period of 1, 2, and 3 h.

3. When studying the effect of NPAg and NPCu combined with ozone-air pre-treatment, it was found that the concentration of 100:100 μg/cm^3 had a fungicidal effect on the test cultures of *Aspergillus flavus*, i.e. treatment with ozone-air mixture enhanced the antimycotic properties of the nanocomplex.

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Article received 21.06.2023

ДОСЛІДЖЕННЯ ФУНГІЦИДНИХ ВЛАСТИВОСТЕЙ ОЗОНОВОЇ ОБРОБКИ, НАНОЧАСТИНОК Ag І Cu ТА ЇХ СУМІСНОЇ ДІЇ НА МОДЕЛІ САНІТАРНО-ЗНАЧУЩОГО ПЛІСЕНЕВОГО САПРОФІТУ *ASPERGILLUS FLAVUS*

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Проведено дослідження з визначення фунгіцидної активності санітарно-значущого контамінанту кормів – представників роду *Aspergillus Mich.* – вид *Aspergillus flavus,* озоновим методом обробки розчину, композицією наночастинок металів Ag і Cu та сумісною дією цих методів. При дослідженні сумісної дії озону та NPAg і NPCu встановлено, що при концентраційному співвідношенні наночастинок 100:100 мг/см^3 має місце фунгіцидна дія щодо музейних штамів тест-культур *Aspergillus flavus*, тобто комбінація з озоном посилила антимікотичні властивості нанокомплексу.