# A COMPARATIVE STUDY OF ANTIMICROBIAL ACTIVITY OF SILVER CLUSTERS AGAINST VARIOUS MICROORGANISMS

**Tatiana A. Rozalyonok*, Yurii Yu. Sidorin**

Kemerovo Institute of Food Science and Technology (University),
Stroiteley blvd. 47, Kemerovo, 650056 Russian Federation

* e-mail rozalyonok@yandex.ru

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**Abstract:** Currently, the problem of micro-organisms resistance to traditional antibiotics, which represents a serious threat to human health, is exposed to close attention. Therefore, the development of alternative antimicrobial agents, including on the basis of silver nanoparticles today becomes relevant. Substantiation of effectiveness of the cluster silver (1–10 nm) in comparison with larger silver nanoparticles is resulted. During the study of domestic and foreign experience of using a cluster of silver, basic mechanisms of its antimicrobial action were analyzed that it may have on organisms. The purpose was to comparatively study the antimicrobial activity of a cluster silver with respect to various microorganisms, including establishment of the minimum inhibitory silver concentration for the following strains: *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger*. The effect of various concentrations of silver clusters (from 0 to 400 ug / ml) contained in a liquid medium, on survival of cultured cells was studied. Using the method of serial dilutions, the difference in effects on silver clusters on growth and reproduction of the following microorganisms was established: bacteria (with a different structure of cell walls: gram-positive-thick-walled, capable to form endospores and gram-negative - thin-walled) and micromycetes (yeast and hyphal). An *in vitro* study of antimicrobial activity of the cluster silver colloidal solution taken at various concentrations and at various exposure times was carried out. Minimum inhibitory concentrations of cluster silver colloidal solution for studied bacteria were determined: opportunistic pathogenic bacterium (*Escherichia coli* and *Bacillus subtilis*) and micromycetes (*Candida albicans* and *Aspergillus niger*).

**Keywords:** Cluster silver, nanosilver, ions, nanoparticles, antimicrobial activity, MIC

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## INTRODUCTION

The antimicrobial activity of silver at all, and silver nanoparticles in particular are of considerable interest in the world today. This is due primarily to the fact that silver nanoparticles exhibit high antibacterial activity against aerobic and anaerobic microorganisms (including antibiotic-resistant strains) [1].

The need for new drugs based on silver nanoparticles is motivated by the fact that there is increasing occurrence of strains of bacteria resistant to traditional antibiotic, posing a serious threat to human health [2].

The antibacterial activity of silver has been known since ancient times [3]. It was established that the silver nanoparticles have a higher activity and show better biocide results against gram-negative bacteria such as *E. coli* [4], than the Gram-positive bacteria.

Studies have shown [5] that the impact efficiency depends on the particle size: he most effective are the nanoparticles, whose size is about 1–10 nm. They have a greater impact on the bacteria than particles in diameter of 1–100 nm.

After analyzing a number of scientific research in this area [6–7], it can be concluded that small-sized nanoparticles are likely to spread more easily than larger ones and this is due to their high toxicity.

Influence of size on antibacterial activity was also investigated by Baker [8]. It appears to him that the antimicrobial properties are related to the total surface area of nanoparticles: smaller particles with a larger surface to volume ratio have greater antibacterial activity. Similar results were also published by Choi and Hu [9].

Thus, the smaller the nanoparticles, the higher permeability they have and behave more active against a number of pathogenic and conditionally pathogenic microorganisms.

Therefore, speaking about the cluster silver, it is necessary to make a clarification that «*cluster silver*» – is a kind of colloidal silver, but with a smaller particle size, their average size, as a rule, is 1–10 nm [10]. And precisely because of their small size, clusters have unusual, unique antimicrobial properties. In addition, a smaller average size of the silver particles increases the efficiency of the silver use and causes aggregation and sedimentation stability of its solutions.

The silver acts on the microbial cell in two steps [2]. The first stage is adsorption by a cell membrane, which has a protective function. The cell remains viable, but some of its functions are violated, for example, division (bacteriostatic effect). The second stage - penetration of ions into cell. When silver is adsorbed on the surface of

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the microbial cell, ions penetrate into the cell and inhibit enzymes of the respiratory chain as well as separate processes of oxidation and oxidative phosphorylation in microbial cells, whereby the cell dies.

In the analysis of the literature it has been noted the fact that compared to the number of studies on the nanosilver infulence on bacteria, silver nanoparticles fungicidal effect is not seen so closely and in such amount as an antibacterial. There is evidence of antifungal action of silver nanoparticles synthesized using various original techniques (mainly biosynthesis), but not any patterns of such action are not observed [11]. In [12–13], the difference was established in the influence of the cluster and ionic silver forms on the growth and reproduction of decomposer microorganisms *Bacillus fastidiosus*, *Lactobacillus sp.*, *Microbacterium terregens*, as well as the effect of different concentrations of the cluster and ionic silver present in the liquid medium on survival of cells of these microorganism cultures was studied.

However none of the work does not contain a comparative study of the effect of different concentrations of silver clusters in relation to opportunistic pathogenic bacteria (with a different structure of cell walls: Gram- positive - thick-walled, able to form endospores and Gram-negative - thin-walled) and micromycetes (yeast and hyphal).

The aim of the work was a comparative study of antimicrobial activity of the cluster silver in relation to a variety of microorganisms, including establishment of minimum inhibitory (bacteriostatic) concentrations (MIC) of silver for the following strains: *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger*.

## MATERIALS AND METHODS

Colloidal solutions of cluster silver mass fraction of 10 000 g/ml) were prepared on the basis of laboratory of Research and Education Center of FSBEI HE Kemerovo Technological Institute of Food Industry (University)" (FSBEI HE "KemTIFI").

Experimental studies were carried out in the laboratory of Research Institute of Biotechnology (Institute of Biotechnology) at FSBEI HE "KemTIFI", in three replications.

The subjects of study were:

– synthesized colloidal solutions of cluster silver (a particle size of 1–10 nm);

– strains of microorganisms, provided by Federal State Unitary Enterprise "GosNIIgenetika": *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger*.

At different stages of the study we used the following chemical reagents and equipment:

– autoclave DGM 80 (Pharma Apparate Handel AG, Switzerland);

– laminar (Jacob Delafon, France);

– upright microscope AxioScope A1 (Carl Ziess AG, Germany);

– inversion microscope Axio Vert A1 (Carl Zeiss AG, Germany);

– thermostat shaking incubator LSI–3016R (Daihan Labtech, Korea);

– spectrophotometer UV 1800 (Shimadzu, Japan);

– analytical scales CAS CAUX 220 (CAS Corporation Ltd, Korea);

– microbiological spatula (Aptaca, Russia);

– bacteriological loop (GOST 492–73);

– distilled water (GOST 6709–72);

– others used domestic and imported reagents have purity of not less than chemically pure.

This study examined the antimicrobial properties of the clustered silver colloidal solutions against the following strains of different microorganisms: opportunistic pathogenic bacterium *Escherichia coli* (representative of Gram-negative organisms - thin-walled) and *Bacillus subtilis* (representative of Gram-positive microorganisms - thick-walled, capable to form endospores) and micromycetes *Candida albicans* (yeast micromycete), *Aspergillus niger* (hyphal micromycete). In the first stage the investigated microorganism cultures grown in fluid medium (Muller-Hinton broth) at temperatures: for *Escherichia coli*, *Bacillus subtilis* – (37 ± 1)°C during 24 h; for *Candida albicans* – (28 ± 1)°C during 48 h; *Aspergillus niger* – (32 ± 1)°C during 72 h [14]. For the cultivation of microorganisms, flat-bottomed conical flasks were used.

After cultivation of microorganisms a cluster silver solution was added in each flask at regular intervals (for 24 hours), whrer by its mass fraction in the medium increased from 0 to 400 mcg/ml [12].

Microscopy was carried out using upright microscope AxioScope A1 Carl Ziess AG and inversion microscope AxioVert A1 Carl Ziess AG, X40 magnification. Microscopy with the use of inversion microscope was carried out in presence of the ethidium bromide fluorescent dye using orange acridine at a ratio of 3: 1. Acridine orange is able to form complexes with pure nucleic acids which fluoresce green when bound to DNA. Ethidium bromide penetrates only in cells with damaged cytoplasmic membrane staining them red. Combined use of these dyes allows to evaluate the ratio of live cells and dead cells: Some of them are stained in green, others - in red. The number of living cells was performed according to the protocols of Methodsin Molecular Biology [12].

At the second phase of the study, determination of *in vitro* antimicrobial activity of the colloidal solution of silver clusters taken at various concentrations (100 ug/ml, 10 ug/ml, 1 ug/ml, 0.1 ug/ml 0.01 ug/ml 0.001mu.g/ml) was carried out at different contact times (0.5 min; 1 min; 5 min; 15 min; 60 min; 120 min; 240 min; 480 min).

During the studies, *in vitro* quantitative method was used to assess the sensitivity of the selected strains of microorganisms to colloidal solutions of silver clusters. Routine of experiment was to use the suspension method (or another method of serial dilutions in broth [15]). The Mueller-Hinton broth was used as the food solution to prepare microbial suspensions of standard strains.

The number of bacteria was determined spectrophotometrically by the optical density of culture

medium at a wavelength λ = 600 nm with UV 1800 spectrophotometer (Shimadzu, Japan).

Based on these results, expressed in CFU ml, the inhibitory activity of the product analyzed in the form of test of its antimicrobial action was assessed.

The index of microorganism ability or inability to reproduce *in vitro* in the presence of a given concentration of a substance having antimicrobial properties, was classified as follows:

– sensibility: microorganism is inhibited by the specified concentration of substance having antimicrobial properties;

– moderate sensibility: microorganism is partially inhibited by the specified concentration of substance having antimicrobial properties; microorganism could react to a higher concentration;

– stability (resistance): microorganism is not fully inhibited by concentration of a substance having antimicrobial properties;

## RESULTS AND DISCUSSION

The antimicrobial activity of silver clusters, as mentioned previously, is related to their extremely small size and large surface area (with respect to volume), which provides a better interaction with microorganisms. Synthesis clustered silver colloids was performed using the modified high-molecular process that involves recovery of silver nitrate with ethylene glycol in the presence of stabilizers such as a polyvidone.

Except for ligand, the stability of nanoparticles is influenced by pH at which the synthesis is carried out. To study the influence of pH on stability of aqueous colloidal solutions, the silver nitrate solution was pre-treated and its pH was determined by NaOH and HCl solutions.

In carrying out the synthesis, the aqueous medium with polyvidone and ethanol, as well as silver nitrate were used. The reduction reaction was carried out in the "drip synthesis" mode with vigorous stirring at 70°C. Under these conditions, ethanol and polyvidone are simultaneously reducers of silver ions (Ag+) and stabilizer (ligand) of silver particles being formed.

In the preparation of colloidal silver, silver content in the aqueous dispersion was 1% (10.000 ug/ml).

The silver particle-size distribution was determined using a laser analyzer and was 1–10 nm (Fig. 1).

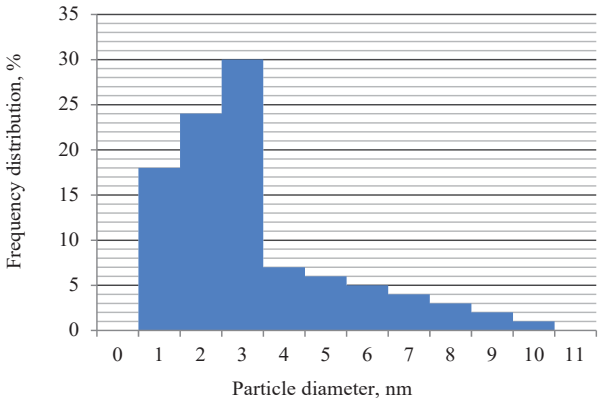


**Fig. 1.** Silver clusters particle size distribution in a colloidal solution.

One of the most important characteristics of the studied colloidal solutions of silver clusters is antimicrobial activity.

At the first stage of the study of antimicrobial properties of the silver clusters colloid solutions, the effect of this colloidal solution with respect to opportunistic pathogenic bacteria was assessed (with a different structure of cell walls: Gram-positive - thick-walled, able to form endospores and Gram-negative - thin-walled) and micromycetes (yeast and hyphal).

Differences in action of silver cluster colloidal solutions in the concentration range from 0 to 400 ug/ml on the studied strains of microorganism *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger* are shown in Fig. 2.

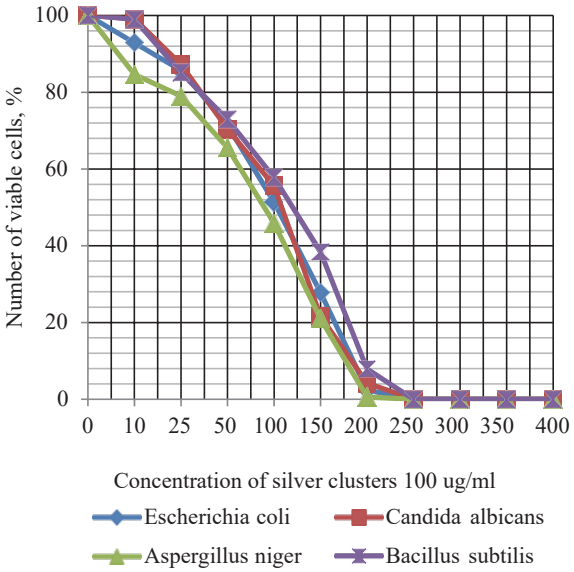


**Fig. 2.** Action of silver cluster colloidal solutions in concentrations ranging from 0 to 400 ug/ml.

Analyzing the data shown in Fig. 2 it can be concluded that at a concentration of 100 ug/ml, he number of viable cells is reduced by about a factor of 2, and at 200–250 ug/ml a complete destruction of these microorganisms occurred.

Based on these data the initial concentration of 100 ug/ml was chosen to further determine the sensitivity of microorganisms to different concentrations of silver clusters, as well as to determine its minimum inhibitory concentration.

During the follow-up study, a method of serial dilutions in liquid medium was used, which allows to set the minimum inhibitory concentration of the preparation for the chosen strain of microorganism.

Based on these results, expressed in CFU/ml, the inhibitory activity of silver cluster colloidal solution was assessed as a test of its antimicrobial action.

The following Fig. 3–7 describe assessment of efficiency of study of the antimicrobial activity of the silver cluster colloidal solution (without dilution), which corresponds to 100 ug/ml; as well as diluted in different ratios 1:10 (10ug/ml); 1:100 (1 ug/ml); 1:1.000 (0.1ug/ml) 1:10 000 (0.01 ug/ml) and 1:100 000 (0.001 ug/ml)

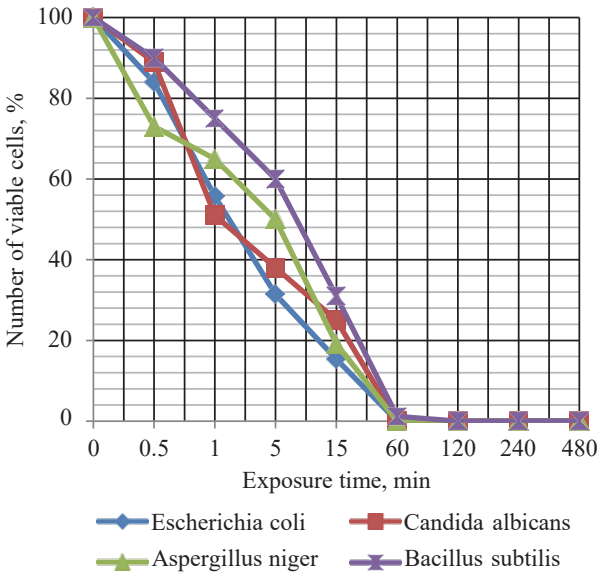


**Fig. 3.** Assessment of efficiency of the cluster silver colloidal solution with concentration of 100 ug/ml (undiluted).

Considering the results obtained (Fig. 3) we can conclude that the silver cluster colloidal solution with concentration of 100 ug/ml showed antimicrobial activity *in vitro*: after 5 minutes of contact the number of living cells decreased by about 2 times, and after 1 hour there was a complete destruction of the considered microorganisms.

As can be seen from the Fig. 4, the silver cluster colloidal solution with concentration of 10 ug/ml showed *in vitro* antimicrobial activity, proportional to increase in a contact time: after 1 hour its efficiency ranged from 70% for *Bacillus subtilis* to 99 % for *Escherichia coli*, *Candida albicans* and *Aspergillus niger*. The presence of Bacillus subtilis spores in the test solution was not established.

It should be noted that the most affected by the silver cluster colloidal solution with concentration of 10 ug/ml is a strain of *Escherichia coli* (a representative of Gram-negative organisms), and the least - *Bacillus subtilis* (representative of gram positive microorganisms capable under adverse conditions to form endospores, possessing radio- or chemo- and thermal resistance).

The observed trend is largely due to the fact that gram positive bacteria, due to differences in the structure of the cell walls are less sensitive to silver nanoparticles in comparison with Gram-negative bacteria [7, 16, 17]. The cell wall of Gram-positive bacteria contains a much greater amount of peptidoglycan and murein, making it negatively charged, which ultimately prevents penetration of silver ions into the cell [18].

Considering the results obtained (Fig. 5) we can conclude that the silver cluster colloidal solution with concentration of 1 ug/ml showed *in vitro* antimicrobial activity: after 1 hour of contact efficiency with respect to *Escherichia coli* was about 90%, with respect to *Aspergillus niger* and *Candida albicans* was about 80 %, as well as with respect to *Bacillus subtilis* was about 70 %. The greatest inhibitory effect the silver cluster colloidal solution had on the growth of the strain *Escherichia coli*, and the least - on *Bacillus subtilis* that confirmed a previously observed trend. The presence of Bacillus subtilis spores in the test solution was not established.

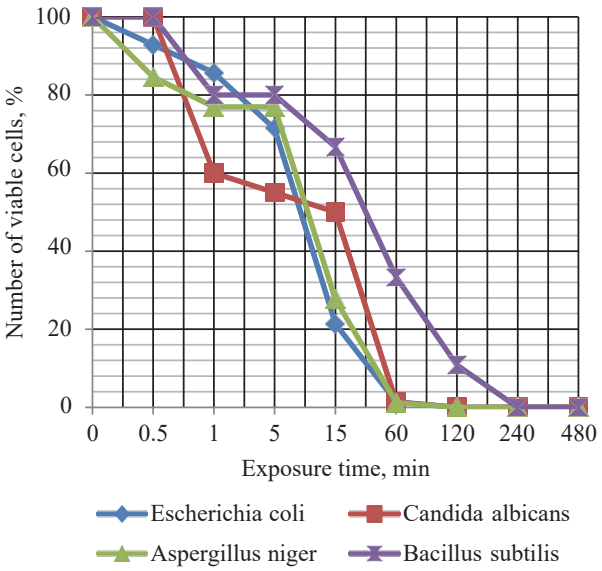


**Fig. 4.** Asessment of efficiency of a cluster silver colloidal solution, 1 : 10 dilution (10 ug/ml).

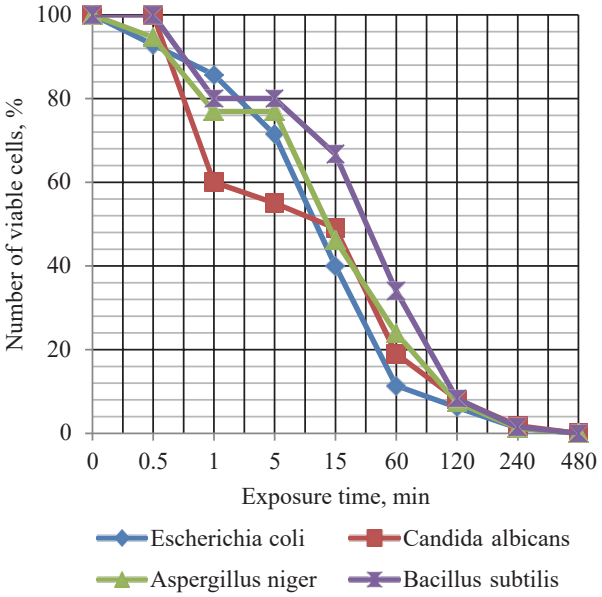


**Fig. 5.** Asessment of efficiency of a cluster silver colloidal solution, 1 : 100 dilution (1 ug/ml).

Analyzing the data obtained (Fig. 6) at 1 : 1000 dilution of the initial colloidal silver solution, *in vitro* antimicrobial activity can be noted: after 1 hour of contact efficiency with respect to *Escherichia coli* was about 40 %, with respect to *Aspergillus niger* and *Candida albicans* was about 30%, as well as with respect to *Bacillus subtilis* was about 20%. The presence of *Bacillus subtilis* spores in the test solution was not established.

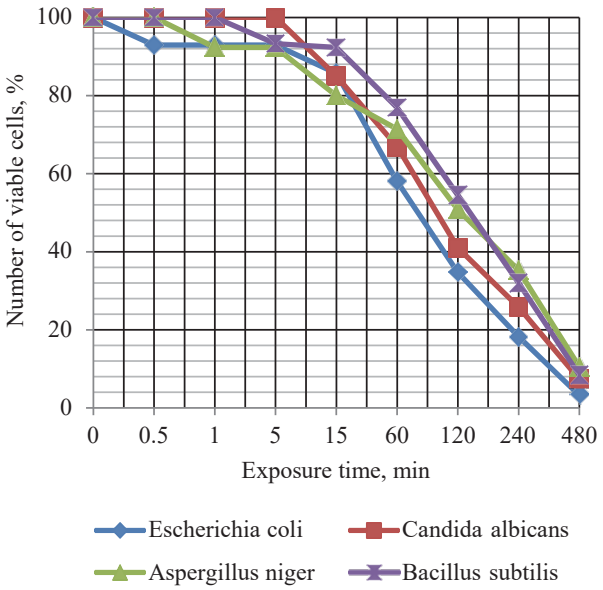


**Fig.6.** Asessment of efficiency of a cluster silver colloidal solution, 1 : 1000 dilution (0.1 ug/ml).

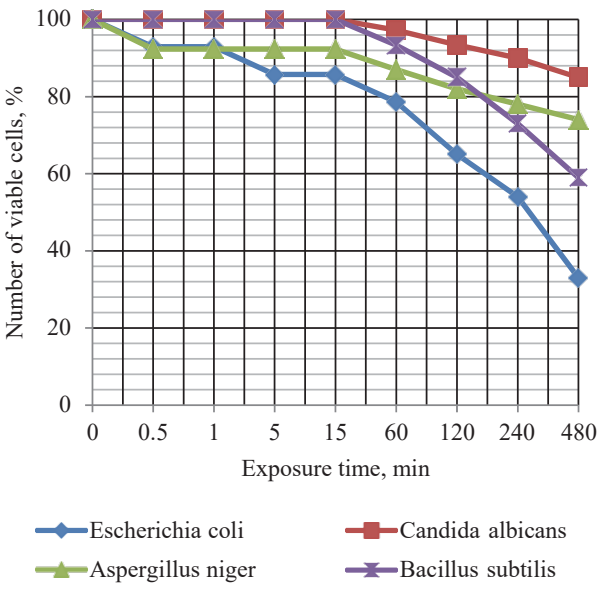


**Fig. 7.** Assessment of efficiency of a cluster silver colloidal solution, 1 : 10000 dilution (0.01 ug/ml).

It should also be noted that at a dilution of 1:10000 (Fig. 7), the silver cluster colloidal solution does not show *in vitro* antimicrobial activity after 1 hour of contact with respect to the analyte microorganisms. At the contact time of up to 15 minutes bacteriostatic effect was observed (i.e., the colony count growth delay) with respect to all strains of microorganisms studied. However, over time, there is still a gradual proportional reduction in the count of living cells, indicating an inhibition activity of this concentration of the colloidal solution of the silver clusters.

During the subsequent dilution (concentration of 0.001 ug/ml) microorganisms were not completely inhibited by the concentration of a substance having antimicrobial properties, which indicates the resistance of microorganisms to this dilution of cluster silver colloidal solution.

Results of assessment of efficiency of cluster silver colloidal solution for test organisms are summarized in Table 1.

**Table 1.** Assessment of the effectiveness a colloidal silver solution for the test microorganisms

| Silver cluster concentration, (ug/ml) | Test microorganism | | | |
|---|---|---|---|---|
| | Escherichia coli | Bacillus subtilis | Candida albicans | Aspergillus niger |
| Without dilution (100) | sensibility | sensibility | sensibility | sensibility |
| Dilution: | | | | |
| 1 : 10 (10) | sensibility | sensibility | sensibility | sensibility |
| 1 : 100 (1) | sensibility | sensibility | sensibility | sensibility |
| 1 : 1000 (0.1) | moderate sensibility | moderate sensibility | moderate sensibility | moderate sensibility |
| 1 : 10000 (0.01) | moderate sensibility | moderate sensibility | resistance | resistance |
| 1 : 100000 (0.001) | resistance | resistance | resistance | resistance |

Thus, analyzing the data obtained in the study we have concluded that the clustered silver colloidal solution exhibits antimicrobial activity from the concentration of 0.001 ug/ml (0.000001% silver content) which is the minimum inhibitory concentration for Gram-negative (*Escherichia coli*) and gram-positive (*Bacillus subtilis*) bacteria.

In contrast, strains of micromycetes *Aspergillus niger* and *Candida albicans* are inhibited more effectively by concentration of 0.01 ug/ml.

The difference in the effect of the cluster silver in the bacteria and micromycetes are associated with the peculiarities of their structure [2, 19, 20]. For example, in contrast to *Escherichia coli*, there is non-reversible interaction of silver ions and cysteine residue at phosphomannose isomerase in *Candida albicans* yeast

under the action of the clustered silver colloidal solution, during which cell wall synthesis interrupts which leads to loss of essential nutrients [13].

In addition, based on experimental data we confirmed that gram negative bacteria (*Escherichia coli*) are less resistant to the action of nanosilver in comparison with Gram-positive bacteria (*Bacillus subtilis*). This feature involves a higher compared with negative bacteria content of peptidoglycan and murein in the cell wall of Gram-positive bacteria, making it negatively charged. The negative charge of the cell wall of Gram-positive bacteria eventually prevents penetration of silver ions into a cell [18]. This pattern was confirmed in scientific studies of foreign authors [2, 4, 21].

## CONCLUSION

The difference in the effects of the silver cluster on growth and reproduction of the following microorganisms was established: bacteria (with a different structure of cell walls: gram-positive - thick-walled, capable to form endospores and gram-negative - thin-walled) and micromycetes (yeast and hyphal). The effect of various concentrations of the silver cluster (from 0 to 400 ug/ml) contained in a liquid medium on survival of cells *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger* was studied. The regularities in *in vitro* antimicrobial activity of the colloidal cluster silver solution at various concentrations (100 ug/ml, 10 ug/ml, 1 ug/ml, 0.1 ug/ml, 0.01 mg/ml, 0.001 mg/ml) at different exposure time (0.5 min; 1 min; 5 min; 15 min; 60 min; 120 min; 240 min; 480 min) with respect to the test microorganism cultures were studied. It is found that the antimicrobial activity of the colloidal cluster silver solution is manifested from the following concentrations: 0.001 ug/ml for opportunistic pathogens (*Escherichia coli* and *Bacillus subtilis*) and 0.01 ug/ml for micromycetes (*Aspergillus niger* и *Candida albicans*).

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**Tatiana A. Rozalyonok**
Postgraduate of the Bionanotechnology Department, Kemerovo Institute of Food Science and Technology (University), Kemerovo, Russian Federation

**Yurii Yu.Sidorin**
Cand.Sci.(Phys.-Math.), Professor and Consultant of Research and Education Center, Kemerovo Institute of Food Science and Technology (University), Kemerovo, Russian Federation