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ANTIBACTERIAL EFFECT OF THINTiO_2:Ag:CuFILM

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Abstract: Infections mediated bymedical devices are a significant social and economic problem. Rapidly developing resistance to traditionally used antibiotics makes the search for new antimicrobial materials a serious contemporary challenge. Recently, the use of nanoparticles and nanomaterials for biomedical research, such as Cu, TiO_2, Ag, etc., has gained more attention due to their unique properties. Nanomaterials are chemicals or materials with at least one dimension of 1 to 100 nm. The thin TiO_2:Ag:Cu films are made by magnetron sputtering under different deposition conditions and with different technological characteristics. They were tested on several bacteria with different cell wall structure: Pseudomonas putida, Salmonella enterica, Bacillus cereus, Staphylococcus epidermidis, Escherichia coli,allarranged in increasing order of resistivity to the film. Inhibition of bacterial growth and propagation is determined by the Koch method and the corresponding optical density measurements. The investigation of the toxicity of nanomaterials is of global importance because of their safety concerns, based on the ability to mechanically destroy live cells. The research and results have shown that some of the nanostructured films have a significant bactericidal effect and might be useful in the future.

INTRODUCTION

The use of conventional products and methods of disinfection has no desired effect in hospitals and medical devices in many cases (Özkaleli et al., 2017) Recently, there has been an increasing interest in the application of nanocomposite materials as antibacterial surfaces that combine mechanical stability and biocompatibility. Different nanomaterials have a multi-purpose mechanism of action on various microorganisms that pose a threat to human health. Modern industrial technologies in material production provide control of nanostructured dimensions and surface morphology of nanomaterials with antibacterial activity (Allahverdiyev et al, 2011; Lee et al., 2015).As different

organisms react differently to the same nanomaterials, it is extremely important to select such material that is toxic to pathogens and not toxic to human tissues.

The development of modern biomedical nanotechnology is related to the development of tissue-like structures and surfaces that favor the development of cells in the multicellular organism, but not of pathogenic bacteria and fungi (Chang et al, 2017; Song et al., 2016). Many modern antimicrobial agents owe their activity to the presence of metal ions such as silver, zinc, titanium, copper and their nanoparticles and oxides (Azadmanjiri et al, 2017; Fakhri et al., 2017; Yousefi et al., 2017).

TiO_2 is widely used for research and in various fields such as modern oral implants, textile impregnation, etc. (Yuranova et al, 2007; Zille et al., 2014). The properties of TiO_2 could be improved by introducing suitable metals into the TiO_2 structure (Dhanapandia et al, 2016;Zaleska et al.,2008). In addition, the presence of an additive may increase the adhesion and mechanical resistance of the thin film to substrates that play a key role in the reliability of the device (Sikong et al., 2008).

The silver-doped TiO_2 has been extensively studied due to its wide use in environmental remediation, catalytic oxidation reactions, antimicrobial protection, and so on. (Zielinska et al 2010; Krejcikova et al 2012). Numerous studies have shown increased activity in both oxygenation and antimicrobial activity of TiO_2 in combination with Ag and Cu (K.D. Kim, 2006). Therefore, this study is focused on the antibacterial activity of TiO_2 thin films doped with Ag and Cu.

MATERIALS AND METHODS

The thin films used are made by the magnetron sputtering (rf Magnetron sputtering) method.

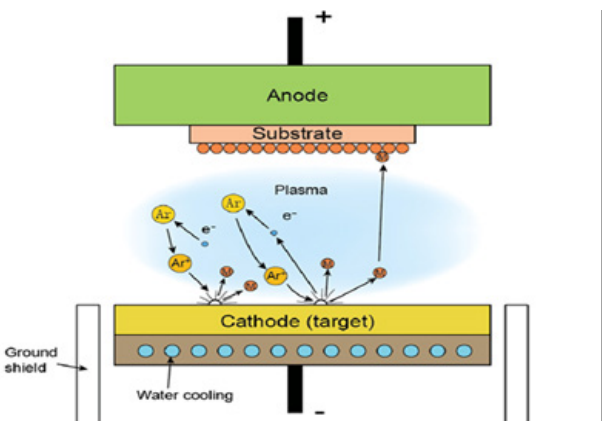


Fig. 1. High-frequency installation for radio frequency (RF) sputtering.

Thin films are sterilized by irradiation for 30 minutes with ultraviolet light. The thin films are then immersed in ethanol and burnt in the flame immediately before use and cooled in sterile condense water on the inside of the petri dish lid. Diffusion test for determination of TiO_2 thin film toxicity with other dopants - 100 μL of bacterial suspension in exponential phase is spread on a solid nutrientmedium with a content specific to the respective bacteria, and then the sterilized and cooled thin film was layed on the surface of the medium. The plates are incubated in a thermostat for 18-24 hours at 37 ° C for *Escherichia coli,* P. aeruginosa and Bacillus cereus and 24 ° C for Pseudomonas putida. Thereafter, the presence of sterile zones, measured in mm, is reported.

The growth inhibition experiment is performed in a sterile 6-well plate. A series of two replicates for bacterial controls, 3 for the thin film films under examination and blank control with a medium and a thin film without bacteria are prepared, the amount of nutrient medium and inoculum in all variants remaining constant. Each hour the optical density is measured, and consecutive ten-fold dilutions are made at 2 hours to count the amount of bacteria in the controls and samples. Each dilution is done in two replicates. The control is inoculum with a clean glass instead of a thin film. Optical density measurements is determined with a Specol 11 (DDR) Spectrophotometer at ƛ= 450 and 610 nm every 60 minutes tillthe 24 hours.

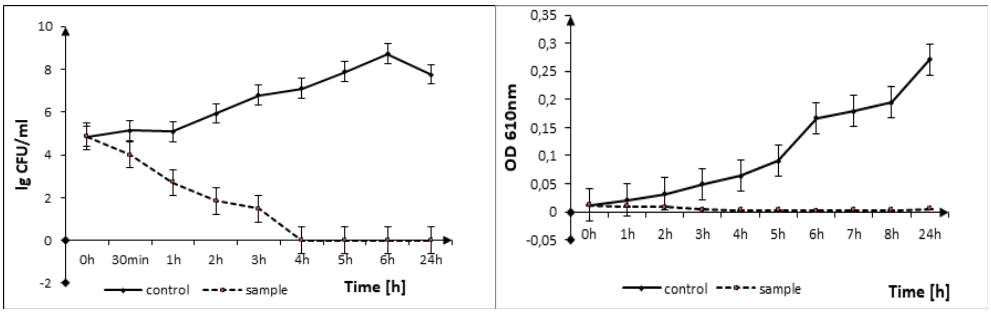
RESULTS AND DISSCUSIONS

Thin films TiO_2: Cu: Ag with a thickness of 60 nm and a spray area of 7850 mm2 of titanium dioxide, SAg = 60 mm^2, SCu = 100 mm^2, were selected to detect growth inhibition of microorganisms. Selected bacteria are of different taxonomic affiliations.

The thin film of TiO_2: Ag: Cu was made in the Central Laboratory for Solar Research and New Energy Sources at the Bulgarian Academy of Sciences precisely by the Magnetron sputtering (rf Magnetron sputtering) method. All experiments were performed in three replicates, standard deviations were inflicted on the graphs.

The microorganisms used are bacteria of different taxonomic nature: *Escherichia coli* 3548 (gram-negative bacteria), *Pseudomonas putida* 1090 (gram-negative bacteria), *Bacillus cereus* 1177 (gram-positive bacteria), *Staphylococcus epidermidis* 3486 (gram-positive bacteria), *Salmonella enterica* 4333 (gram-negative bacteria).

Figure 2 shows the growth of *B.cereus* in the presence of a thin film of TiO_2:Ag:Cu; SAg=60 mm^2, SCu=100 mm^2.

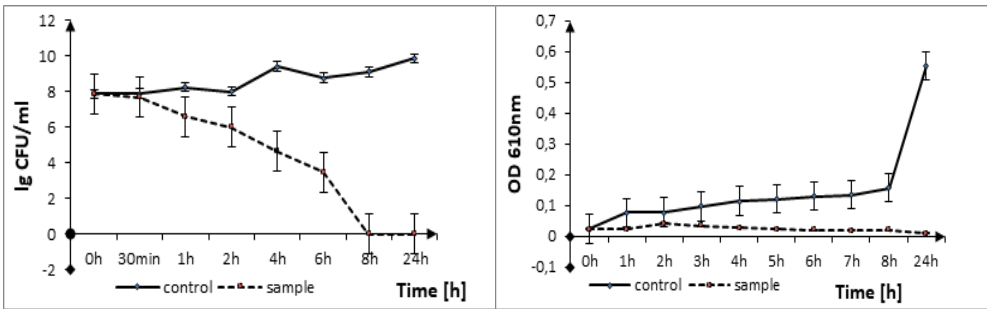


A **B**

Fig. 2. Development of B.cereus in the presence of thin film of TiO_2: Ag: Cu.
A) The Koch method; **B)** Optical density measurements

The growth curves in the presence of thin TiO_2: Ag: Cu films are on figure 2, where the dense line represents the bacterial amount in the control without TiO_2: Ag: Cu, and the dotted line represents the amount of surviving cells in the TiO_2: Ag: Cu film sample, both in logarithmic scale. The inhibition in dynamics was determined by the Koch method (Figure 2A), respectively, the optical density measurements (Figure 2B). Standard deviations were inflicted on the graphs.

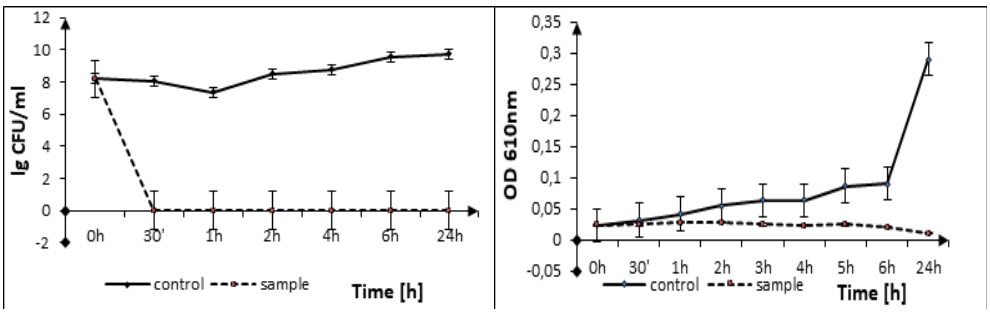
Both methods used show a similar growth curve - rapid bacterial destruction within 4 hours in the presence of thin film. Koch's 24-hour studies show that there are no surviving cells, suggesting the bactericidal effect of the thin film on the vegetative cells and spores of *Bacillus*.



A **B**

Fig. 3. Growth of S. epidermidis in the presence of thin films TiO_2: Ag: Cu
A) The Koch method; **B)** Optical density measurements.

The effect of the tested films on another Firmicutes bacterium, such as *S. epidermidis,*are shown in Figure 3A (Koch method) and 3B (optical density measurement). Standard deviations were inflicted on the graphs.In this case, delayed cell death is observed up to 8 hours and no survival is observed after 24 hours. The result may be due to irregular clumps of cells and surface biofilm, as well as its lower growth rate (0.2), compared to that for *Bacillus* (0.4), characteristic of this bacterium. As it is well known, cells in biofilm are more resistant to environmental factors.



A **B**

Fig. 4. Growth of P.putida in the presence of thin films TiO_2: Ag: Cu
A) The Koch method; **B)** Optical density measurements.

Figure 4 shows the bactericidal effect of the same thin TiO_2: Ag: Cu films on *P. putida* growth determined by the cultivation method (Figure 4A). The results obtained from the optical density measurements at λ = 610 nm are shown in (Figure 4B). Standard deviations were inflicted on the graphs. The bactericidal effect on the Pseudomonas strain is the fastest and the result is visualized by a scanning electron microscope (JOEL JSM 5510) as destruction of bacterial cells at two optical magnifications in Figure 5 (A and B). The dark areas around the bacteria show the organic matter leaked out of the damaged cells and burned by the electron beams. Damage to the cell membrane leads directly to cell leakage that causes cell death. This demonstrates the mechanical destruction of the cells caused by the nanoparticles released by the film and the chemical destruction by reactive oxygen species (ROS), formed on the surface of the film- during theinteraction with bacteria, as has been found by other authors Sondi and Salopek-Sondi (2004), Zavilgelsky et al. (2011).

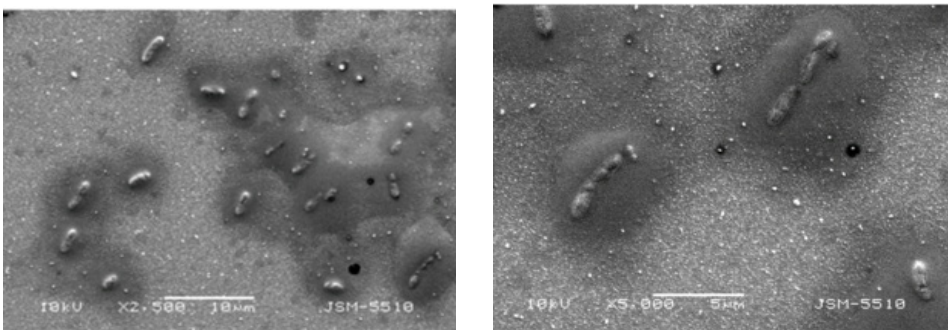
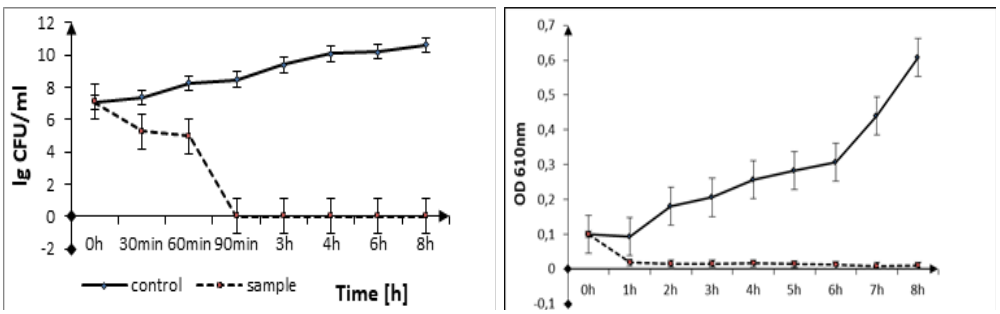


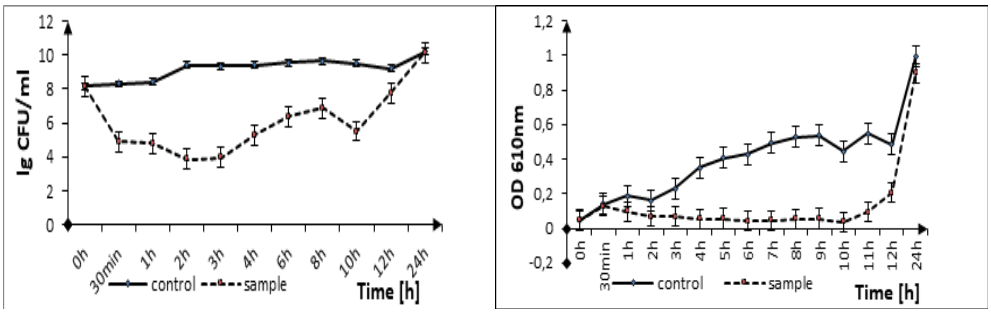
Fig. 5. View of P.putida cells on thin TiO2: Ag: Cu film by scanning electron microscope with different magnifications: **A)** 2500 times;**B)** 5000 times.



A **B**

Fig. 6. Growth of S.enterica in the presence of thin films TiO2: Ag: Cu
A) The Koch method; **B)** Optical density measurements.

The influence of thin TiO_2: Ag: Cu films on *S. enterica* growth is presented in Figure 6A (Koch method) and 6B (optical density measurements). Standard deviations were inflicted on the graphs. The optical density decreases very rapidly as early as the first hour, while live S. enterica cells are established by the Koch method for 90 minutes and are below 10 live cells per ml of culture medium. They are presented only till the eighth hour for clarity. The bactericidal effect of the investigated thin films on the bacteria, represented by Gracilicutes, *S. enterica* and *Pseudomonas* sp. is faster than Firmicutes, which implies a higher sensitivity of these bacteria to the nanocomposite material.



A **B**

Fig. 7. Growth of E. coli in the presence of thin films TiO_2: Ag: Cu
A) The Koch method; **B)** Optical density measurements

The same thin film has only a bacteriostatic effect on *E. coli.* High growth retention was observed from the beginning to the 3rd hour of the experiment. The results presented in Figure 7 (A-Classic Method of Cultivation) clearly show this. Standard deviations were inflicted on the graphs.

At the 24 hours of the experiment, good growth of *E. coli* was observed. By contrast, all other bacterial strains are completely deactivated.

The average growth rate of controls (test bacterial strains without nanomaterials) was calculated from the exponential phase as 0.404; 0.279; 0.214; 1, 472 and 0.283, respectively, for *B.cereus, S.epidermidis, P.putida, S.enterica* and *E. coli.*

The bacteriostatic effect and adaptation of *E. coli* to the tested nanomaterials can be explained by the ability of this bacterial species to activate special transport pumps for the export of nanoparticles or ions from the cell. This fact is a possible stress-response mechanism, as other researchers have reported (Stoyanov et al., 2003). They have discovered that *E. coli* is able to control the copper and silver content in the cells and also the disposal of these ions from the cytoplasm of its cell.

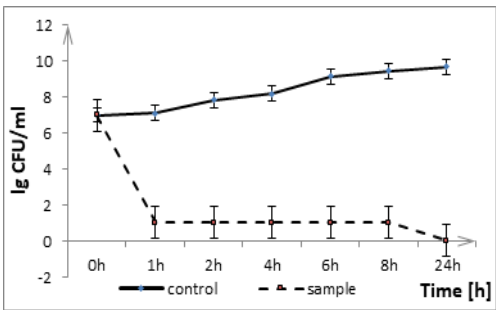


Fig. 8 Development of *E. coli* in the presence of a 80 nm thin film with deposition area SAg 190 / SCu 180 mm^2.

The curve in Figure 8 shows the bacterial death after the 8th hour despite the dark conditions in which it was placed. Therefore, the bactericidal effect in this case is due not so much to the illumination but to the toxic effect of metals deposited on the film of titanium dioxide - copper and silver. A decrease in the number of cells was observed up to 24 hours. This nanostructured film has a 100% bactericidal effect on E. coli since no bacterial growth has occurred at 24 h at 37 ºC. The area of TiO_2 is greater than that of Ag 39 times more, and 42 times that of Cu. Obviously, in this case, the major toxic metal nanoparticles for the bacteria are the deposited copper and silver. The results obtained are of interest for the future deepening of the research with these nanocomposite thin films.

CONCLUSIONS

From our research and the results obtained, it can be concluded that some of the nanostructured films have a significant bactericidal effect and might be useful in the future.

Thin films composed of TiO_2: Ag: Cu of 60 nm thickness and 7850 mm^2 titanium dioxide spray area; of silver SAg = 60 mm^2, of copper SCu = 100 mm^2 have a pronounced bactericidal effect against *B.cereus* to the 4th hour and against *S. epidermidis* at 8 hours. The bactericidal effect against *Pseudomonas* was demonstrated at 30 minutes and in *S. enterica* at the 90th minute of the experiment. Scanning Electron microscopic images detected cell membrane damages and leaking of the cell content. The bactericidal effect of 60 nm film TiO_2: Ag: Cu on *S. enterica* and *Pseudomonas* sp. is faster than *S. epidermidis* and B.cereus, which means that these bacteria are more sensitive to the nanocomposite material. Unlike *E.coli,* all other bacterial strains are completely deactivated by the 24th hour.

The amount of copper and silver added in the tested films is essential. The sensitivity of the bacteria to TiO_2: Ag: Cu films of 60 nm SAg = 60 mm^2, SCu = 100 mm^2 is in the following order: *Pseudomonas putida, Salmonella enterica, Bacillus cereus, Staphylococcus epidermidis, E. coli.*

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REFERENCES

1. Allahverdiyev M., Abamor S., Bagirova M., Rafailovich M. (2011) Antimicrobial effects of TiO_2 and Ag_2O nanoparticles against drug-resistant bacteria and leishmania parasites. *Future Microbiol*, vol. 6, pp. 933–940.
2. Azadmanjiri J., Wang J., Berndt C., Kapoor A., Zhu M., Ang M., Srivastava K. (2017) Influence of charged defects on the interfacial bonding strength of tantalum- and silver-doped nanograined TiO_2. *Phys Chem Chem Phys*.19(19):11881-11891.
3. Dhanapandian S., Arunachalam A., Manoharan C. (2016) Highly oriented and physical properties of sprayed anatase Sn-doped TiO_2 thin films with an enhanced antibacterial activity. *Applied Nano Science*. vol. 6, no. 3, pp. 387-397.

4. Dimova-Malinovska D., Angelov O., Nichev H., Pivin J. (2007) ZnO:H thin films for room temperature selective NH_3-sensors. *J Optoel Adv,* 9: 248-251.
5. Fakhri A., Tahami S., Naji M. (2017) Synthesis and characterization of core-shell bimetallic nanoparticles for synergistic antimicrobial effect studies in combination with doxycycline on burn specific pathogens. *J Photochem Photobiol B.* 169:21-26.
6. Kim D., Han N., Lee B., Kim T. (2006) Formation and characterization of Agdeposited TiO_2 nanoparticles by chemical reduction method, *Scripta Mater* 54 143–146.
7. Krejcikova S., Matejova L., Koci K., Obalova., L Matej Z., Capek L. (2012) Preparation and Characterization of Ag-Doped Crystalline Titania for Photocatalysis Applications. *Appl. Catal. B,* vol. 111−112, pp.119−125.
8. Lee K., Choi S., Jang I., Choi Y. (2015) Improved osseointegration of dental titanium implants by TiO2 nanotube arrays with recombinant human bone morphogenetic protein-2: a pilot in vivo study. *Int J Nanomedicine,* vol. 10, pp. 1145–1154. doi: 10.2147/IJN.S78138.
9. Sikong L., Damchan J., Kooptarnond K., Niyomwas S. (2008) Effect of doped SiO2 and calcinations temperature on phase transformation of TiO_2 photocatalyst prepared by sol – gel method. *Songklanakarin Journal of science and Technology*, , vol. 30, no. 3, pp. 385-391.
10. Sondi I., Salopek-Sondi B. (2004) Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. *J Colloid Interface Sci.,*275: 177-182.
11. Stoyanov V., Magnani D., Solioz, M. (2003) Measurement of cytoplasmic copper, silver, and gold with a lux biosensor shows copper and silver, but not gold, efflux by the CopA ATPase of *Escherichia coli. FEBS Lett.*; 546: 391- 394.
12. Wang S., Zhu W., Yu P., Wang X., He T., Tan G., Ning C. (2015) Antibacterial nanostructured copper coatings deposited on tantalum by magnetron sputtering. *Res Paper,3*0 (3): 120-125.
13. Yousefi M., Dadashpour M., Hejazi M., Hasanzadeh M., Behnam B., Guardia M., Shadjou N., Mokhtarzadeh A. (2017) Anti-bacterial activity of graphene oxide as a new weapon nanomaterial to combat multidrug-resistance bacteria.*Mater Sci Eng C Mater Biol Appl.7*4:568-581.
14. Yuranova T.,Laub D., Kiwi J. (2007) Synthesis, activity and characterization of textiles showing selfcleaning activity under daylight irradiation *Catal. Today,* vol.122, pp. 109–117.
15. Zaleska A. (2008) Doped-TiO2: A Review. Recent Pat. Eng., vol. 2, pp.157−164.
16. Zavilgelskii B, Kotova V, Manukhov V. (2011) Titanium Dioxide TiO_2 Nanoparticles Induce Bacterial Stress Response Detectable by Specific Lux B*iosensors. Nanotechnologies in Russia;* 6(5–6): 401–406.
17. Kowalska E., Sobczak W.,Lacka I.Gazda M., Ohtani B., Hupka J. (2010) Silver-Doped TiO_2 Prepared by Microemulsion Method: Surface Properties, Bio- and Photoactivity. *Sep. Purif. Technol*., vol. 72, pp. 309−318.
18. Zille A., Almedida L., Amoria T., Carneiro N., Esteves F., Silva J., Souto, P. (2014) Application of nanotechnology in antimicrobial finishing of biomedical textiles. *Materials Research Express* 1, 032003 2053-1591/14/032003+38$33.00 IP Address: 193.137.16.114.
19. Zkaleli M., Erde, A. (2017) Factors promoting Staphylococcus aureus disinfection by TiO_2, SiO_2 and Ag nanoparticles. *The Online Journal of Science and Technology,* vol. 7, no. 2, pp. 51-55.