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INTERACTION BETWEEN *BACILLUS CEREUS* AND NANOSTRUCTURED THIN FILMS OF ZINC OXIDE AS A TRANSDUCER ELEMENT FOR BIOSENSING APPLICATION

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ABSTRACT
The purpose of this report is the interaction between Gram-positive bacteria of genus *Bacillus* and nanostructured thin film ZnO projected as a transducer element for the engineering of nanobiosensors. For this purpose, a periodic culture of *Bacillus cereus, strain ATCC11778* was browght in a direct contact with a ZnO pad of film thickness of 2 μm for 24 hours. All processes are monitored by combination of optical measurement, cultivation method, fluorescence microscope and scanning electron microscope observation. The obtained results demonstrate unambiguously that the nanostructured ZnO film influences the cell growth and division rate. We proved that the film influence depends on the chemical composition of the film and the method for its preparation. For example, the thin film of nanostructured ZnO prepared by sol-gel dip coating is more toxic and inhibits the cell division than the film prepared by magnetron sputtering deposition. We found also that the pH of the bacterial solution is a crucial factor for the strength of ZnO effect.

Key words: *Bacillus cereus, nanostructured ZnO thin films, dips coating, magnetron sputtering.*

INTRODUCTION

The bacterial sensor is a device that detects, transmits and records information regarding a biochemical or physiological change in the environment. Technically, it integrates an electronic transducer element prepared from thin film or nanostructured material (1). The choice of material(typically semiconductor) is important for the sensitivity and environmental stability of the biosensor. Recently zinc oxide (ZnO) and other metal oxides are widely applied in the construction of biosensors (2-5). ZnO receives considerable attention, because of its unique optical, semiconductor, piezoelectric, and magnetic properties. It is also a biocompatible material with a high isoelectric point of ~ 9.5 which makes it suitable for the absorption of proteins. However, nothing is known about the interaction between a nanostructured transducer element of ZnO and the detected bacteria. Some metal oxide nanoparticles with diameter < 100 nm have toxic effect on the bacterial suspension (6). Franklin and co-authors report for a toxic effect of ZnO nanospecies when they are in a direct contact with *P. subcapitata* bacterial suspension. They have found that the toxicity of ZnO particles is essential due most probably to the dissolved Zn^{2+} ions (7). Brayner and co-authors have reported that *E. coli* cells are damaged after contact with ZnO showing Gram-negative triple membrane disorganization (8). Another group reports that the concentration of ZnO nanoparticles is more important than the particle size, and the reason for the toxic effect

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is a damage of the cell wall (9). They infer that ZnO particles are effective for inhibiting of both Gram-positive and Gram-negative bacteria and have an antibacterial activity against spores that are otherwise highly resistant (10). The high temperature treatment of ZnO particles leads to lower antibacterial activity. ZnO nanotubes deposited on a glass are used as a working electrode to fabricate an enzyme-based biosensor for glucose, phenol and other pollutants through enzymes immobilization (5, 11).

The interaction between *E. coli* and ZnO nanorods is studied by transmission electron microscope (TEM) and thedestruction of bacterial membranes is examined. It is proven that the formation of H_2O_2 per se may not play a role for the cell-membrane destruction. Instead of this, the release of Zn^{2+} ions by the ZnO surface and their interaction with the membranes is assumed as an alternative mechanism of antibacterial activity (10).

In our study, we prove the interaction of nanostructured thin films of ZnO with a bacterial suspension. In most cases the films inhibit the growth of *Bacillus* cells in the exponential phase. This effect depends on the preparation method for thin films. For example, the ZnO films prepared by magnetron sputtering activate the rate of cell division between the 9th and 24th hour in comparison with the control experiment. On other hand, the ZnO films prepared by sol-gel dip coating do not activate the cells division and the bacterial suspensions have the same density as the control experiment for the period between 9th and 24th hour.

MATERIALS AND METHODS

2.1 Preparation of nanostructured thin films.

Two sets of ZnO samples are used. The first set ZnO thin films were deposited on glass substrates by r.f. magnetron sputtering from a ZnO ceramic target (100 mm disc) in atmospheres of Ar (0.5 Pa) or Ar (0.5 Pa) + H_2 (0.1 Pa) at substrate temperatures 500 and 400 ^oC, respectively. The sputtering was carried out at r.f. power of 180 W. The thickness of deposited films is about 600 nm (13). The other set of samples was prepared by sol-gel dip coating. The sol is based on Zn acetate, 2-methoxyethanol and monoethanolamine (MEA) precursors. The film deposition was carried out on glass substrates (ISO-LAB-Germany). Each deposited layer was dried up in air at 60°C for 30 minutes. The final annealing is carried out at 500°C for 1 hour. The thickness of the films is about 1μm.They have a polydisperse structure with a mean grain size of about 20-32 nm (14).

2.2. Preparation of bacterial culture

The inoculum was prepared from a single 24-h colony of *Bacillus cereus,* strain11778, in a rich medium (ISO 10712) and precultured after 12 h in a poor medium. The overnight culture was refreshed for 1 h before the experiment in a poor medium (ISO 10712). The cells were cultivated in aerated light conditions in a sterile 6 well TC plate with a lid at 150-rpm and temperature 30 ˚C. The light conditions were realized by an energy saving lamp Ecoline Eco 32 (20WE27 warm light 2700 K) at a distance of 30cm. The samples of bacterial cultures in contact with ZnO thin films and of the control experiment (no ZnO films) were collected at every 3 hours. An additional control sample (no ZnO films) was kept in dark conditions, wrapped with aluminium foil to be compared with the samples under light illumination. The optical densities of all samples were measured every 3 h by Specol 11 at λ=600 nm in test medium. Immediately ten-fold dilutions were prepared, inoculated and cultivated on a rich solid medium in dark conditions at 30˚C to determine the quantity of colony-forming units (CFU/ml).

The bacterial abundance ranged from 10^5 to 10^8 cells/ml in poor and rich medium during the experiment (ISO 12072). ZnO thin films were collected from the bacterial suspension after 24 h cultivation, dried at room temperature in a sterile Petri dish for a month, covered with thin Au film, observed and analysed by scanning electron microscope (SEM). Some nanofilms were washed out with a de-ionized water and dried at room temperature for a month before the study by SEM. After the 24th h, the final pH of cultural medium was measured. All experiments were performed in three measurements.

RESULTS AND DISCUSSION

The gram-positive bacteria *Bacillus cereus* fad during the exponential phase between the 3th and 9th hours when they are in a direct contact with the nanostructured ZnO thin films prepared by the above methods. These two types of films possess almost same the inhibition effect **(Fig. 1)**. One can conclude that, at this stage of cell division there is no

difference in the influence of the ZnO nanofilms (prepared by different methods: magnetron sputtering and dip coating) on *Bacillus cereus*.

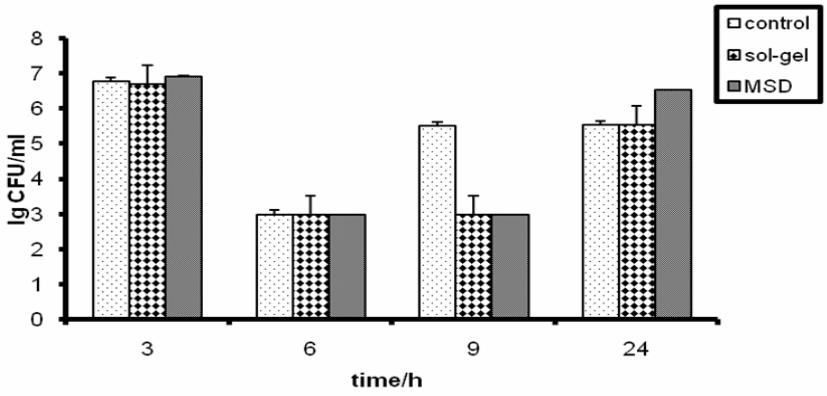

Figure 1. Logarithm from the Number of CFU/ml counted at 24 h on a test medium. Control – control sample, sol-gel – ZnO nanofilm obtained by liquid sol-gel dipcoating, MSD – by vacum magnetron sputtering deposition.

Figure 1 shows the logarithm of CFU/ml of *Bacillus cereus* (active cells) versus the time of incubation in the presence of ZnO films. It is seen that the amount of living cells decrease about 1000 times within the 5^{th} – 6^{th} hour, due to sporulation. This amount remains further constant up to the 9^{th} hour. Then it starts growing to reach nearly the initial amount of bacteria at about 12^{th} hour and does not change up to the 24^{th} hour. The ZnO films strongly decrease the number of active bacteria on them seen from the comparison with the control at the 9^{th} and 24^{th} hour.

Cells collected from the 6th and 9th h of cultivation failed to grow till 24th h and colonies do not appear in rich solid medium till the 48 h of cultivation. Only the control sample formed visible colonies at the 9th h of experiment for less than 24 h in a solid medium, and they could be considered as active dividing cells. The cells possibly form spores after the 5th h of cultivation. This may be a reason for the lower bacterial quantity determined at the 6th and 9th h of the experiment. The spores are inactive and more time is needed to form visible colonies. That is the reason why they appeared after 48 h of cultivation in rich medium and in dark conditions (*see* **Fig. 2**).

Figure 2 depicts the colonies, appearing secondary up to the 48^{th} hour of cultivation of the petri dishes from the spores (figure 1). Their number decreases naturally with time due to exhausting of the medium. It is seen that ZnO films by sol-gel give more spores than the control experiment due to more unfavorable conditions for the bacteria on the films. Indeed the SEM photographs reveal the bacteria covered with nanoparticles (possibly artifacts of the ZnO films). In contrast the Zno films by magnetron sputtering give fewer spores.

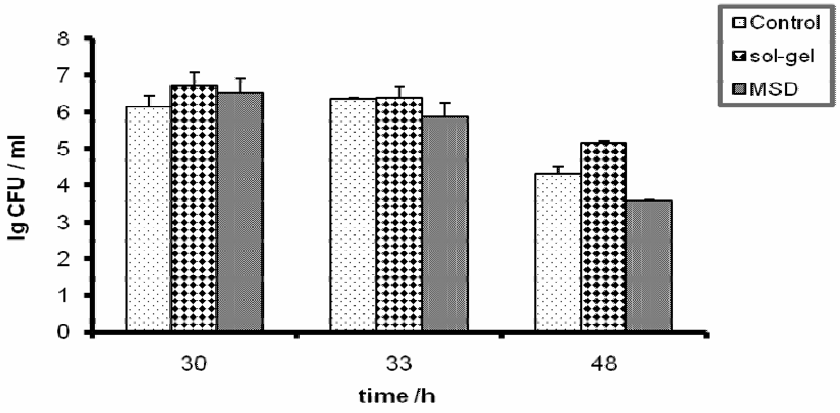

Figure 2. Logarithm number of additionally appearing colonies at 48 h of cultivation on a rich medium.

In all experiments, the optical density of bacterial suspension in the presence of nanostructured ZnO films is higher in comparison with that in the control experiment, and the cells division of *Bacillus cereus* is not inhibited after the 9th h of cultivation. This phenomenon is demonstrated by the classical cultivation methods. In solid media we count not only the active cells, which are enumerated after 18-24 h of cultivation at 30˚C, but as well the spores. In these conditions, it seems that only active dividing cells appear at first and those that can be repaired from spores have formed visible colonies after 48 h. This is illustrated in the **Fig. 3.**

Figure 3 plots the optical density as an impression of total number of bacterial cells (active plus spores) versus the time of incubation. The general trend is to increase their number, because the bacteria are in the stage of exponential growth. At the same time, the ZnO films obtained by sol-gel method give always a smaller number of bacteria due to their harmful effect on them. In contrast, the ZnO films by magnetron sputtering stimulate the cell division, which increase the number of bacteria with respect to the control.

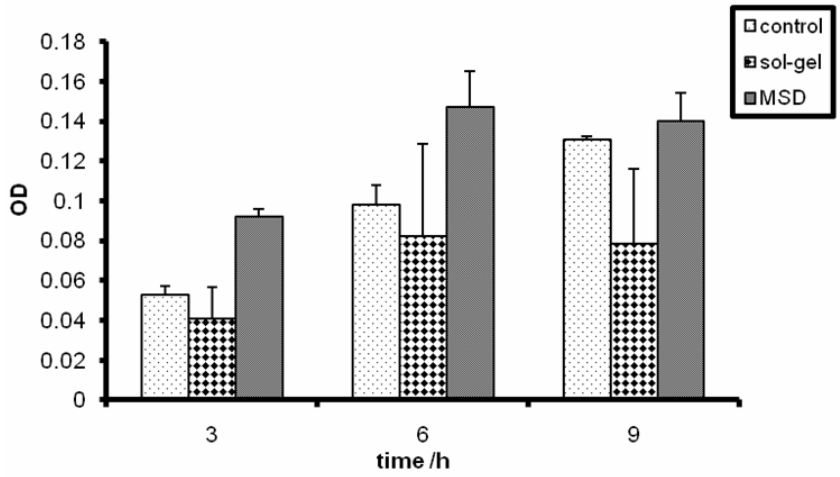

Figure 3. Optical density of bacterial suspensions in the presence of ZnO thin films obtained by different methods. The dot are recorded at a wavelength λ=610 nm.

The bacterial growth curve for the sample with ZnO thin film is comparable with the one for rich and poor liquid media with ZnO films. The cells in a poor medium with ZnO thin films are dividing faster than those in the control experiment at same conditions (poor medium). This is possible, if Zn^{2+} ions are dissolved from the nanostructured films during the investigation process. Those Zn^{2+} ions satisfy the bacterial needs of microelements, which are omitted in a poor test medium (ISO 10712). On **Fig. 4** are shown two SEM micrographs of nanostructured ZnO prepared by the two different methods. The analysis reveals that they have different morphological structure surface, which is important for the film stability in aqueous environment. The ZnO prepared by the magnetron sputtering deposition possess a smooth surface and therefore less area in contact with the suspension in comparison with the films obtained by the sol-gel method. This difference plays an important role for the interaction with bacteria. Nanoparticles from the sol-gel film can detach easier in the solution. They are toxic for the cell (7-9) and that is the reason why the bacterial cells are less visible on the surface of thin films in this case.

We register decreasing of pH of the liquid medium with bacteria to 5.75 during the incubation process. Probably it is due to the consummation of dissolved Zn^{2+}, because the bacterial concentration is highly increased after the 9th hour (if it is in contact with the ZnO thin film prepared with magnetron sputtering deposition).

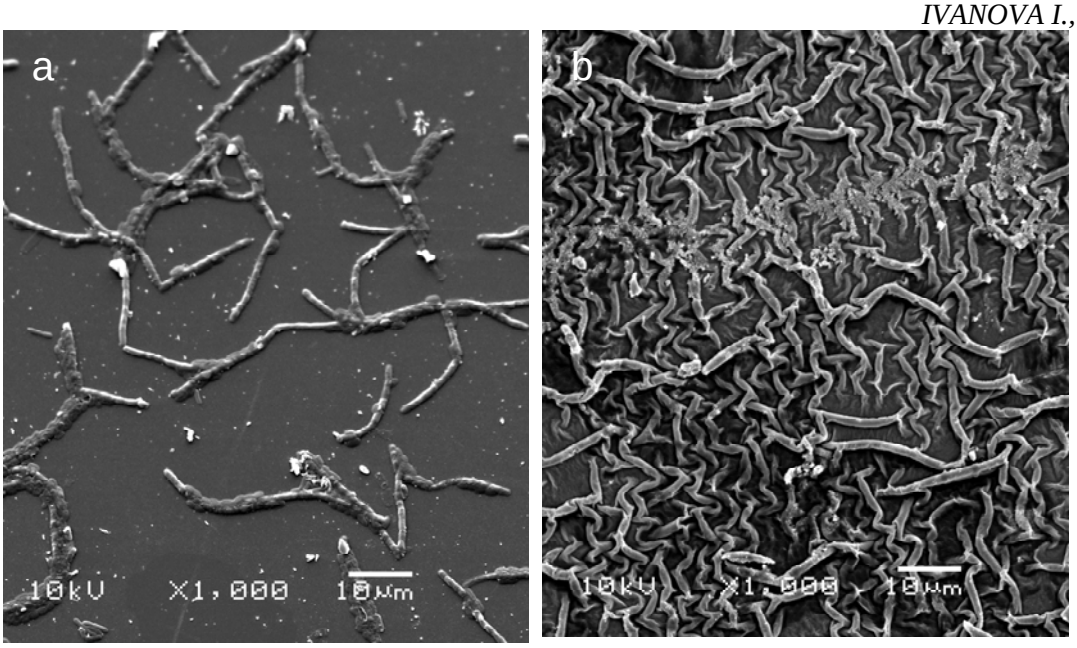

Figure 4. SEM of ZnO thin films obtained by (a) magnetron sputter deposition and (b) dip coating method after 24 h of cultivation in bacterial suspension. The bar scale is 10 μm. The smaller the patterns in (b) represent the native ganglia by sol-gel process. They are superimposed on the rod-like bacteria *Bacillus cereus* and thus exaggerate the picture.

CONCLUSION

This investigation reports the physiological influence of nanostructured ZnO thin films on the cell division of *Bacillus cereus*. This influence is strongly dependent on the method for film preparation. Our results prove unambiguously that the thin ZnO films obtained by magnetron sputtering are not toxic for the *Bacillus cereus* suspension for a period after the 9^{th} h. This method seems more reliable for the preparation of suitable ZnOthin films as a transducer element in the biosensor rather than the method of sol-gel processing.

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