Romanian Biotechnological Letters *Vol. , No. x,*

ORIGINAL PAPER

The effect of titanate nanotubes towards moderately halophilic bacteria

Received for publication, July, 6, 2016
Accepted, November, 9, 2017

SIMONA NEAGU[1], SILVIU PREDA[2], MARIA ZAHARESCU[2], MASAHIRO KAMEKURA[3], ROXANA COJOC[1], MĂDĂLIN ENACHE[1*]
[1]*Institute of Biology Bucharest of the Romanian Academy, Splaiul Independenţei 296, P.O. Box 56-53, Bucharest 060031, Romania*
[2]*Institute of Physical-Chemistry "Ilie Murgulescu" of the Romanian Academy, Splaiul Independenţei 202, Bucharest 060021, Romania*
[3]*Halophiles Research Institute, 677-1 Shimizu, Noda-shi, Chiba-ken 278-0043, Japan*
**Address for correspondence to: madalin.enache@ibiol.ro*

Abstract

This work focuses on the interaction of titanate nanotubes with moderately halophilic bacteria belonging to genera Virgibacillus and Bacillus, indicating the biologically active properties of these nanostructured materials. Subsequently, this activity of the obtained nanosystems on the above mentioned bacterial cells is determined and discussed. The results show a significant dependence of the functional performances on the system's composition and morphology. In particular, the antimicrobial activity of investigated nanotubes is correlated with the preparation methods in various experimental conditions. To the best of our knowledge, this is the first report on the investigation of the effect of titanate nanotubes synthesized under different conditions on halotolerant microorganisms, Virgibacillus halodenitrificans and Bacillus subtilis, isolated from a Neogene-dated subterranean salt rock. The data from this study argued also for the importance of the knowledge of the interactions between these new materials and halophilic microorganisms since environments which hosting these microorganisms are intensively used for recreational and balneology purpose.

Keywords: Antibacterial, halophilic, nanotubes, saline, titanate

1. Introduction

Nanoscience represents a modern approach in frontier research investigations. Although several methods were developed to synthesize a high range of nanomaterials like thin films, nanotubes, nanosphere, etc. (E. KATZ & I. WILNER, 2004 [1]) of wide application spectra, little is known about their impact on the environments (S. MERCIU & al., 2009 [2]). The synergy of bio- and nanotechnologies lead to a rapid development of a blend of various nanomaterials exhibiting protein, enzyme, DNA properties and nanoparticle general features (E. KATZ & al., 2003 [3]; E. KATZ & I. WILLNER, 2004 [1]). The green nanotechnologies are focused on developing a solution to current biotopes challenges. Nanoparticles have several possible environmental applications, including self-cleaning coatings that limit the detergents requirement, pollution-control agents, air and water filters, new-generation photovoltaic cells, etc. Surfaces coated with a titanium oxide film with nano dimensions can prevent water contamination with pathogenic microorganisms by photocatalysis (M. GARTNER & al., 2008a [4]; 2008b [5]). Previous literature data showed that several organic pollutants present in soil (e.g. chlorinated hydrocarbons and biphenyl, pesticides, etc.) could be broken down from polluted soil by using iron constituted nanoparticles (S. MERCIU & al., 2009 [2]).

The unusual chemical (A. KUMAR & al., 2003 [6]), electronic (G. PETO & al., 2002 [7]), optical (A. KROLIKOWSKA & al., 2003 [8]), and photoelectrochemical (N.

CHANDRASEKHARAN & P.V. KAMAT, 2000 [9]) properties of nanomaterials raised the interest for synthesizing these systems of broad utilization. In the presence of UV light, titanium dioxide generated reactive oxygen species, such as hydroxyl and superoxide radicals, that supported the degradation of several organic compounds and bacteria (C. VACAROIU & al., 2009 [10]). Recent studies revealed that titanium dioxide covered with silver nanoparticles increased its bactericidal activity against *E. coli* (K.D. KIM & al., 2006 [11]), while titanium dioxide blended with carbon nanotubes increased disinfectant properties towards *Bacillus cereus* spores (V. KRISHNA & al., 2009 [12]). Silver-doped titanium dioxide nanoparticles inactivated *B. cereus* spores from aluminum and polyester surfaces (A. VOHRA & al., 2005 [13]) and killed airborne bacteria and molds when incorporated into an air filter (A. VOHRA & al., 2006 [14]). On the other hand, sodium titanate thin film characterized by a porous network and sodium titanate nanotubes with antibacterial activity towards a methicillin-resistant *Staphylococcus aureus* strain mainly designated for the development of novel antibacterial implants was recently presented (Y. INOUE & al., 2010 [15]). Nanotubes, tubular structures with an outer diameter of up to 100 nm, represent an alternative to spherical nanoparticles of wide applications in bio- and nanotechnologies. These structures have distinct inner and outer surfaces, which can be chemically or biochemically functionalized (P. KOHLI & C.R. MARTIN, 2006 [16]). Consequently, the inner part of the nanotube can be loaded with a particular biochemical payload, but imparting chemical features to the outer surface that renders it biocompatible. In this study, we tested the antibacterial activity of titanate nanotubes against moderately halophilic bacteria *Virgibacillus halodenitrificans* and *Bacillus subtilis* taking into account the fact that these bacterial strains have been isolated from an area frequently used for halotherapy, namely from subterranean salt mine Unirea, located in Slanic, Prahova, Romania (M. ENACHE & al., 2012 [17]). Since salted environments are widely used in Romania for recreational activity (V.A.C. BULGAREANU, 1996 [18]; M. ENACHE, 2011 [19]) it is of high interest to investigate the impact of new materials like titanate nanotubes on halophilic microorganisms that populated such areas.

To the best of our knowledge, this is the first report on the investigation of the effect of titanate nanotubes synthesized under different conditions on halotolerant microorganisms, *Virgibacillus halodenitrificans* and *Bacillus subtilis*, isolated from a Neogene-dated subterranean salt rock.

2. Materials and Methods

Nanotubes material

The titanate nanotubes were prepared by hydrothermal synthesis using commercial Aeroxide P25 TiO_2, according to the method published before (S. PREDA & al., 2013 [20]). The hydrothermal treatment was performed in the presence of 10 M NaOH solution at 140°C for various time durations (from 24 and 48 h), after ultrasound homogenization for 10 minutes. The mass ratio between TiO_2 and NaOH was 0.04. The reaction slurry was separated by centrifugation and washed alternately with distilled water, 0.1N HCl solution, down to pH ~6. The samples were air dried at 110°C, 12 h. The samples were divided in two batches, one was kept as is, and the other one was subsequently thermally treated at 400°C for 1 h. Experimental conditions for titanate nanotubes synthesis are summarized in Table 1.

The structural characteristics of the synthesized nanotubes were determined by electron microscopy using a JEOL-TEM 200 CX electron microscope. The TEM samples were prepared by dispersing the nanotubes powders in alcohol and collecting a small drop of this dispersion on holey carbon grids for TEM.

Isolation of halophilic bacterial strains

For testing the bacterial growth in the presence of titanate nanotubes, were used strains of moderately halophilic bacteria isolated from hypersaline habitats from Slanic Prahova county, Romania, Cultivation was carried on MH medium, containing 100 g/l NaCl, 7 g/l $MgCl_2x6H_2O$, 9.6 g/l $MgSO_4x7H_2O$, 0.36 g/l $CaCl_2x2H_2O$, 2 g/l KCl, 0.06 g/l $NaHCO_3$, 0.026 g/l NaBr, 1 g/l glucose, 5 g/l proteose peptone, and 10 g/l yeast extract (A. VENTOSA & al., 1989 [21]). The medium was solidified with 10 g/l agar. The pH range of the medium was 7.0-7.2. The preliminary characterizations of the strains followed the previously described microbiological methods (R. COJOC & al., 2009 [22]).

16S-rRNA gene amplification and sequencing

The cultivated bacterial cells were harvested in stationary phase and the total DNA was isolated using glass beads. The cells were suspended in TEN buffer (10 mM Tris pH 8, 1 mM EDTA, 0.1 M NaCl), and disrupted by vortexing for 30 minutes at 20˚C in the presence of glass beads. The lysates were treated with an equal volume of PCI solution (Phenol: Chloroform: Isoamyl alcohol, 25:24:1 v/v/v), and were stirred for 15 minutes. To the aqueous phase were added 1/10 volume of 3 M ammonium acetate and three volumes of 99.5% ethanol. The total DNA was collected by centrifugation, washed with ethanol 70% and dissolved in TE buffer (10 mM Tris pH 8, 1mM EDTA).

The 16S rRNA genes were amplified by PCR (polymerase chain reaction) with the following forward and reverse primers 5'-ATTCCGGTTGATCCTGCCGG-3' and 5'-AGGAGGTGATCCAGCCGCAG-3'. The amplified DNA fragments were dephosphorylated using Shrimp Alkaline Phosphatase (SAP) and the sequences generated using the Abi Prism BigDye Terminator Sequencing Kit (Applied Biosystems) and the forward and reverse primers respectively (5'-ATTCCGGTTGATCCTGCCGG-3' and 5'-GACTACCAGGGTATCTAATC-3') were analyzed by a 310 Genetic Analyzer (Applied Biosystems).

Measurement of antibacterial properties of nanotubes

For further investigations, the strains were cultivated on the MH medium containing 10% NaCl. The antibacterial activity of nanotubes was determined by the plate counting method. 9 mg of nanotubes were resuspended in 40 ml of sodium/potassium phosphate buffer (pH 7) and inoculated with 2 ml of each bacterial strain. The mixture was incubated at 37^oC under stirring conditions at 200 rpm. At certain time intervals, samples of 1 ml were taken, serially diluted and mixed with MH medium agar, poured into Petri dishes and incubated at 37^oC for 48 h and the number of colonies, resistant remaining bacteria, was counted.

Electron microscopy of bacterial cells

Cells were observed by electron microscopy following the adapted method of Hayat (M.A. HAYAT 1972 [23]). The bacterial cells incubated with different types of nanotubes were harvested by centrifugation at 9,500 rpm and incubated at 4^oC for 16 h (overnight) with 0.2 M sodium cacodylate-HCl buffer containing 3% glutaraldehyde. The cells were washed with 0.1 M of the same buffer prior and after being mixed with 2% agar. The cells were further fixed in 1% OsO_4 by incubation for 16 h at 4^0C, and dehydrated with 1:1 (v/v) alcohol: propylene oxide. The cells were further embedded in epoxy resin EPON 812 (Fluka). After polymerization, thin sections obtained with LKB ultramicrotome were contrasted by Raynolds method (E.S. REYNOLDS, 1963 [24]) using lead citrate and uranil acetate, and visualized with a transmission electron microscope EM-125 (Selemi—Ukraina) at 75 kV electrons acceleration.

3. Results and discussion

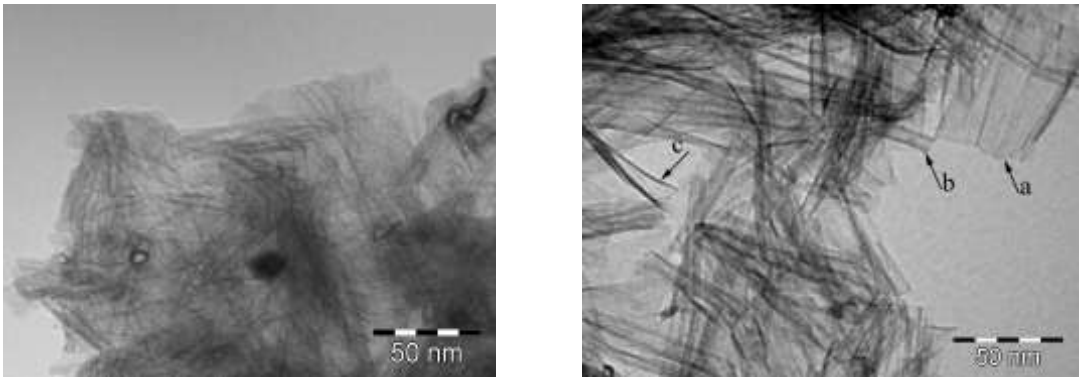
Nanotubes material

Titanate nanotubes were obtained by hydrothermal synthesis under different temperatures and reaction times; post-hydrothermal reaction thermal treatment was applied in order to determine the thermal morphology stability (Table 1). All synthesized nanotubes were dried at 110^{o}C for 12 h, but only the samples Ib and IIb were supplementary annealed at 400^{o}C.

The morphological characteristics of the resulted nanotubes were assessed by electron microscopy (Fig. 1, samples Ia and IIa). The results showed that although most of the obtained nano-particles were tubular-shaped, other morphologic types were also formed; in Fig. 1b, different morphologies are indicated by arrows (a - nanosheets, b - nanotubes, c – scrolling sheets). The secondary phases were higher in sample Ia and Ib, obtained after a shorter hydrothermal treatment period (24 h) as compared to samples II.

Table 1. Experimental conditions for titanate nanotubes synthesis

Sample	Experimental conditions				Product description
	Reaction composition	Time of reaction (h) / temperature (°C)	Drying (h)	Annealing (h)	
			110°C	400°C	
Ia	TiO_2 + NaOH	24/140	12	-	Nanotubes having L≈ 50 nm and Ø ≈ 8-9 nm
Ib				1	
IIa		48/140		-	
IIb				1	



a) b)

Figure 1. TEM image of the titanate nanotubes prepared by hydrothermal method: a) 24 h (sample Ia); a) 48 h (sample IIa); assignment of the marks in Fig.1 b: a = sheet, b = nanotube, c = scrolling sheets

The shape and size of the synthesized nanotubes are similar with those reported in the literature (S. PREDA & al., 2013 [20]; J. NADOR & al., 2014 [25]).

Taking into account the differences in the morphology of the obtained titanate nanotubes, different antibacterial effects could be expected.

Bacterial strains

We selected two halophilic bacterial strains, 1/9 and 1/13 for further investigation. These bacterial strains were cultivable on medium supplemented with sodium deoxycholate, in the presence of high concentrations of NaCl, up to 3M (strain 1/9) and 2M, (strain 1/13). They were sensitive to chloramphenicol and erithromicin. The partial 16S rRNA gene sequence of strain 1/9 was 100% identical with *Bacillus subtilis* AJ276351, while that of strain 1/13 showed 99% similarity with *Virgibacillus halodenitrificans* AY543168 in Blast analysis. In accordance, a phylogenetic analysis based on 16S rRNA sequences (Fig. 2) showed a more

distant relation with *B. halophilus*. The morphological and physiological characteristics of the two strains are summarized in Table 2. These two strains appear to be halotolerant bacteria.

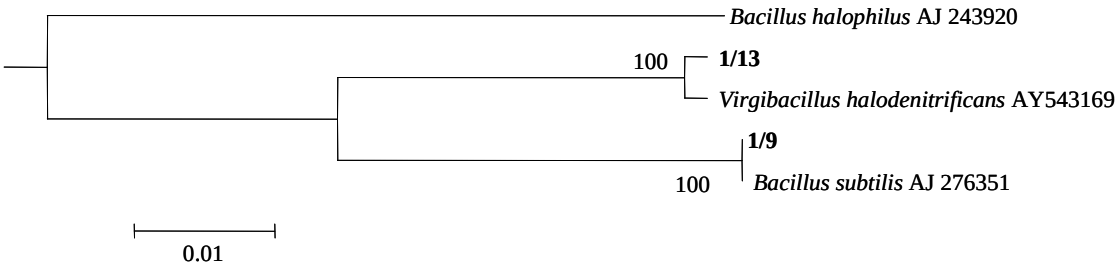


Figure 2. Phylogenetic tree reconstructed from 16S rDNA sequences using neighbor-joining method, indicating the positions of the tested strains relative to *Virgibacillus* and *Bacillus* species.

Table 2. Characterization of investigated bacterial strains

	Strain 1/9	Strain 1/13
Morphology	rod	coccus
Gram staining	+	+
Origin	Salt crystal (salt mine)	Salt crystal (salt mine)
Hydrolysis of:		
starch	+	-
gelatin	+	-
tween 80	+	+
olive oil	-	-
casein	+	-
CMC	+	-
RBBxylan	+	-
Antibiotic resistance		
Neomycin	-	+
Penicillin	-	+
Anysomicin	-	+
Erythromycin	-	-
NaDeoxycholate	+	+
Chloramphenicol	-	-
Upper limit of NaCl concentration for growth	3M	2M
Growth period at 12^0C (weeks)	after 7	after 12

Electron microscopic investigation

The cells of the investigated strains incubated in the absence or presence of nanotubes for 24 h were investigated by electron microscopy (Fig. 3). In the absence of nanotubes, the bacterial cells presented a normal morphological aspect, their majority having oval or circular shapes, characterized by high electron density forms (Fig. 3a). Other pictures (Fig. 3b, f) revealed the digestion of the cell wall followed by the extrusion of the cellular content. In contact with sample IIa, (nanotubes obtained after 48 h reaction time and dried at 110^oC) the cells of *V. halodenitrificans* presented a porous cell wall which allowed the extrusion of cell content. These cells were characterized by massive gas vacuoles (Fig. 3d). The presence of sample Ia (nanotubes obtained after 24 h reaction time, and dried at 110^oC) induced the formation of modified cell clusters, residual cells and cell ghosts (Fig. 3e, f).

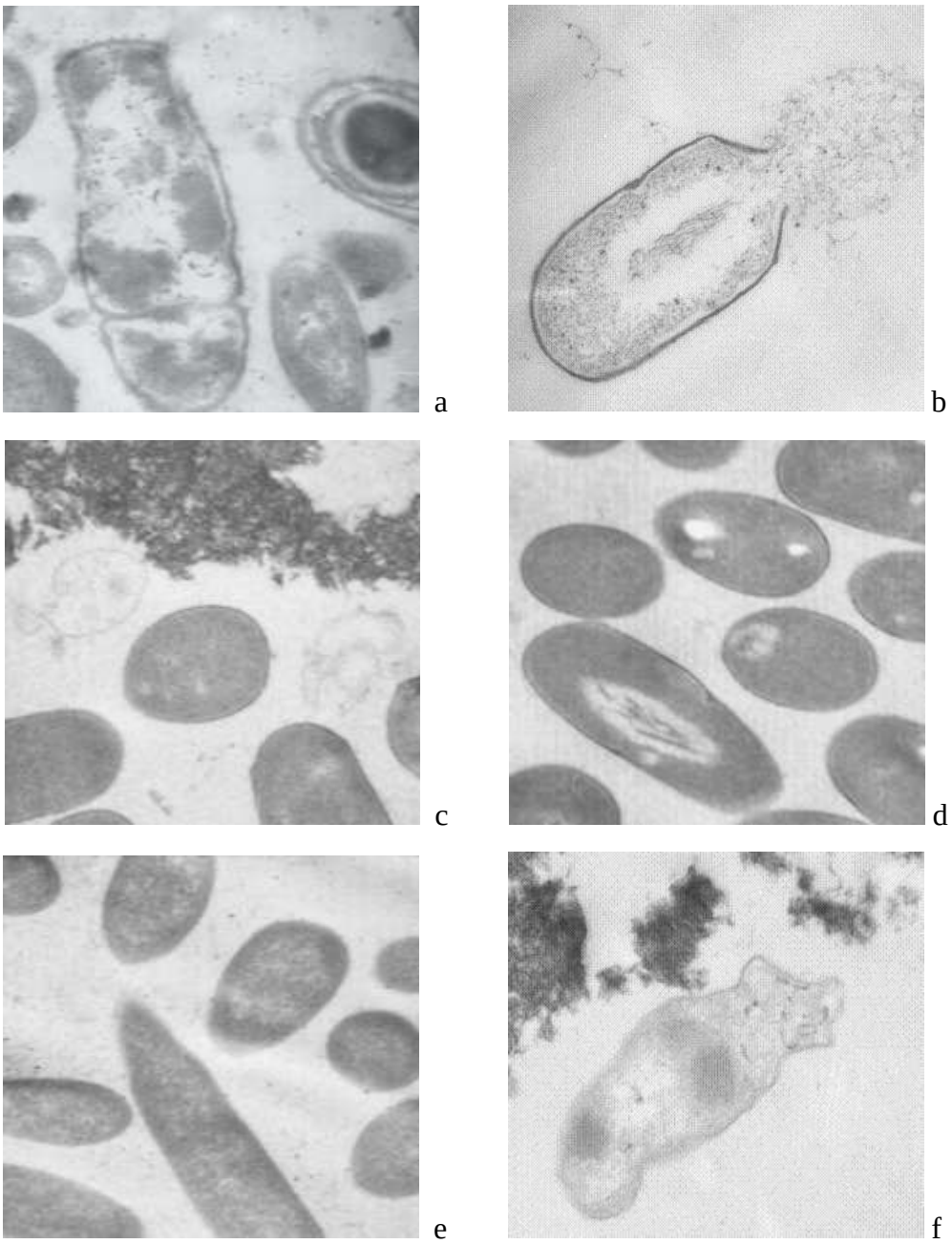


Figure 3. *V. halodenitrificans* without nanotubes (a) and in the presence of sample IIa (b, c and d) and sample Ia (e and f)

The effect of nanotubes on bacterial cells

The two strains, *Bacillus subtilis* 1/9 and *Virgibacillus halodenitrificans* 1/13 were investigated for their viability when interacted with the obtained titanate nanotubes. During the first several hours of incubation, viabilities of the two strains varied considerably depending on experiments and nanotubes used. These differences could be related either to the composition of the cell wall of the microorganisms, or to the variation of conditions applied for nanotubes synthesis. Moreover, the possible presence in the mixture for the synthesis of nanotubes of scarce amounts of amorphous (unstructured) titanate powder with antibacterial activity cannot be completely excluded. It was noticed that after incubation with

nanotubes for several hours, colonies of both strains were translucent compared to those of cells not incubated with nanotubes. This could be due to the presence of high amounts of exopolysaccharides synthesized by these microorganisms, representing a protection mechanism to prevent their interaction with the nanotubes.

After incubation for 24 h, viabilities of the two strains were relatively reproducible. The antibacterial properties of nanotubes were shown to be influenced by the synthesis method (Fig. 4 and 5). The results revealed that thermal treatment of nanotubes obtained after 48 h reaction time (Fig. 4 and 5) showed a similar answer from the investigated strains in opposite with nanotubes resulted after 24 h reaction time. In this last case, it appears that the strain of *B. subtillis* showed a good resistance compared with the strain of *V. halodenitrificans* (sample Ib). The antibacterial activity of nanotubes prepared at 48 h of reaction time without thermal treatment proved that the investigated strains behavior could be associated with the composition of their external cell wall.

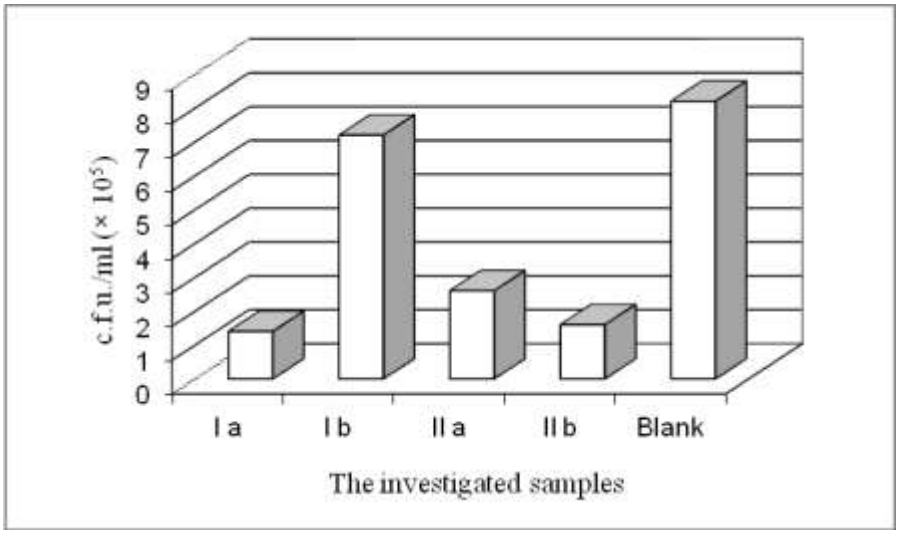


Figure 4. Total number of c.f.u./mL counted after 24 h of incubation of *Bacillus subtillis* 1/9 with the four preparations of titanate nanotubes

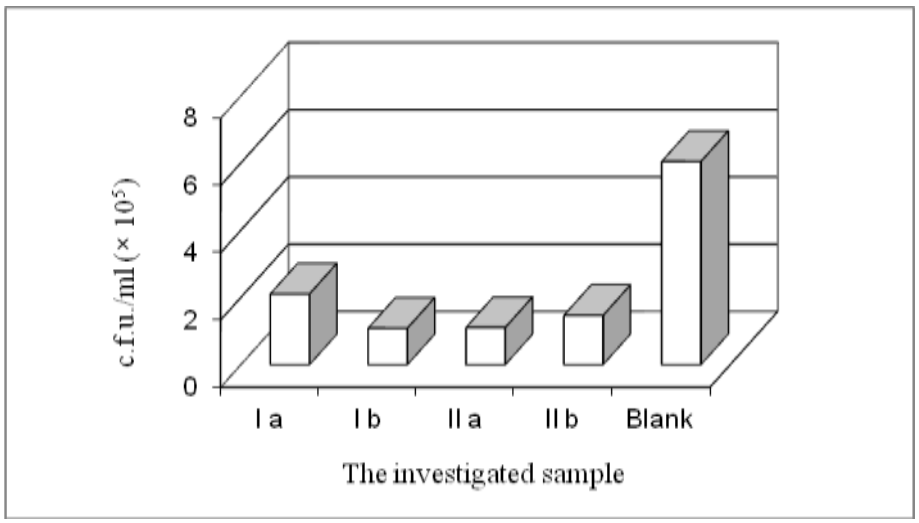


Figure 5. Total number of c.f.u./mL counted after 24 h of incubation of *Virgibacillus halodenitrificans* 1/13 with the four preparations of titanate nanotubes

4. Conclusion

The data reported here demonstrated the antibacterial properties of titanate nanostructured materials towards moderately halophilic microorganisms belonging to genera *Virgibacillus* and *Bacillus*. The thermal treatment (conducted at 400 °C for 1 h) and a synthesis reaction time of 48 h led to an increased antibacterial activity of titanate nanotubes. We also consider that the impact of nanotubes on the investigated moderately halophilic microorganisms could be assimilated to a bacteriocin-like mechanism. Thus, modifications detected at the level of the cell wall must be probably due to alteration of lipids and the protein membrane profile supported this hypothesis. Taking into account the increasing society development and claims for the new products the novel interactions between human and biological ecosystems are expected. In this frame, the accelerating rhythm of the introduction of the new materials like nanotubes in daily life claims for high interest for the investigations (M. ENACHE & al., 2015 [26]) about their interactions with microorganisms and other life forms. The data from this study argued also for the importance of the knowledge of the interactions between these new materials and halophilic microorganisms since environments which hosting these microorganisms are intensively used for recreational and balneology purpose.

5.Acknowledgements

The authors express their warm gratitude to dr. Akinobu Echigo and dr. Hiroaki Minegishi from BioNanoElectronic Research Center, Toyo University, Kawagoe, Japan for help with DNA sequencing data of the investigated strains. We also thank dr. Aurelia Brezeanu for help with electronic microscopy techniques. Parts of this study were supported by the Program Partnerships in Priority Domains, PN-III-P2-2.1-PED-2016-139 (9PED/2017), funded by MEN-UEFISCDI, and the Project RO1567-IBB05/2015, Institute of Biology Bucharest of the Romanian Academy.

References

1. E. KATZ, I. WILLNER, Integrated nanoparticle–biomolecule hybrid systems: synthesis, properties, and applications, *Angewandte Chem. Intl. Ed.*, 43, 6042, 6108 (2004).
2. S. MERCIU, C. VĂCĂROIU, R. FILIMON, G. POPESCU, S. PREDA, C. ANASTASESCU, M. ZAHARESCU, M. ENACHE, Nanotubes with biological activity in media with high salt concentration, *Biotechnol. & Biotechnol. Equip.*, 23, 827,831 (2009).
3. E. KATZ, A.N. SHIPWAY, I. WILLNER, Biomaterial-nanoparticle hybrid systems: Synthesis, properties and applications, G. SCHMIDT (ed.) *Nanoparticles - From Theory to Applications*, Wiley-VCH, Weinheim, pp. 368-421 (2003).
4. M. GARTNER, C. ANASTASESCU, M. ZAHARESCU, M. ENACHE, L. DUMITRU, T. STOICA, T.F. STOICA, C. TRAPALIS, The Simulation in the Real Conditions of Antibacterial Activity of TiO, (Fe) Films with Optimized Morphology, S. MATHUR & M. SINGH (eds.), T. OHJI & A. WERESZCZAK (volume eds.) *Nanostructured Materials and Nanotechnology II*, Ceramic Engineering and Science Proceedings, Volume 29, Issue 8, John Wiley & Sons, Inc., Hoboken, New Jersey, pp. 67-76 (2008a).
5. M. GARTNER, C. TRAPALIS, N. TODOROVA, T. GIANNAKOPOULOU, G. DOBRESCU, M. ANASTASESCU, P. OSICEANU, A. GHIȚĂ, M. ENACHE, L. DUMITRU, T. STOICA, M. ZAHARESCU, J.Y. BAE, S.H. SUH, Doped sol-gel TiO_2 films for biological applications, *Bull. Kor. Chem. Soc.*, 29, 1038,1042 (2008b).
6. A. KUMAR, S. MANDAL, P.R. SELVAKANNAN, R. PARISCHA, A.B. MANDALE, M. SASTRY, Investigation into the interaction between surface-bound alkylamines and gold nanoparticles, *Langmuir*, 19, 6277, 6282 (2003).
7. G. PETŐ, G.L. MOLNÁR, Z. PÁSZTI, O. GESZTI, A. BECK, L. GUCZI, Electronic structure of gold nanoparticles deposited on SiO_x/Si (100), *Mat. Science and Engineer.: C*, 19, 95, 99 (2002).

8. A. KROLIKOWSKA, A. KUDELSKI, A. MICHOTA, J. BUKOWSKA, SERS studies on the structure of thioglycolic acid monolayers on silver and gold, *Surface Science*, 532, 227, 232 (2003).
9. N. CHANDRASEKHARAN, P.V. KAMAT, Improving the photoelectrochemical performance of nanostructured TiO_2 films by adsorption of gold nanoparticles. *The J. of Phys. Chem. B,* 104, 10851,10857 (2000).
10. C. VĂCĂROIU, M. ENACHE, M. GARTNER, G. POPESCU, M. ANASTASESCU, A. BREZEANU, N. TODOROVA, T. GIANNAKOPOULOU, C. TRAPALIS, L. DUMITRU, The effect of thermal treatment on antibacterial properties of nanostructured TiO_2(N) films illuminated with visible light, *World J. Microbiol. Biotechnol.*, 25, 27, 31 (2009).
11. K.D. KIM, D.N. HAN, J.B. LEE, H.T. KIM, Formation and characterization of Ag-deposited TiO_2 nanoparticles by chemical reduction method, *Scripta Materialia* 54, 143, 146 (2006).
12. V. KRISHNA, S. PUMPRUEG, S.H. LEE, J. ZHAO, W. SIGMUND, B. KOOPMAN, B.M. MOUDGIL, Photocatalytic disinfection with titanium dioxide coated multi-wall carbon nanotubes, *Process Safety and Environm. Protect.*, 83, 393, 397 (2005).
13. A. VOHRA, D.Y. GOSWAMI, D.A. DESHPANDE, S.S. BLOCK, Enhanced photocatalytic inactivation of bacterial spores on surfaces in air, *J. Ind. Microbiol. Biotechnol.*, 32, 364, 370 (2005).
14. A. VOHRA, D.Y. GOSWAMI, D.A. DESHPANDE, S.S. BLOCK, Enhanced photocatalytic disinfection of indoor air, *Appl. Catal. B: Environm.*, 64, 57–65 (2006).
15. Y. INOUE, M. UOTA, T. TORIKAI, T. WATARI, I. NODA, T. HOTOKEBUCHI, M. YADA, Antibacterial properties of nanostructured silver titanate thin films formed on a titanium plate, *J. Biomed. Mat. Res.*, 92, 1171, 1180 (2010).
16. P. KOHLI, C.R. MARTIN, Smart nanotubes for biotechnology, *Curr. Pharma. Biotechnol.*, 6, 35, 47 (2005).
17. M. ENACHE, G. POPESCU, T. ITOH, M. KAMEKURA, Halophilic microorganisms from man-made and natural hypersaline environments: physiology, ecology and biotechnological potential, H. STAN-LOTTER & S. FENDRIHAN (eds.) *Adaptation of Microbial Life to Environmental Extremes*, Springer, Wien New York, pp. 173-197 (2012).
18. V.A.C. BULGĂREANU, Protection and management of anthroposaline lakes in Romania. *Lakes & Reservoirs: Research & Management*, 2, 211, 229 (1996).
19. M. ENACHE, Aspects of the biological complexity in salt lakes, M. ENACHE (ed.). *Biological complexity under macro, micro, nano aspect*, Ed. Acad. Rom., pp. 117-126 (*in Romanian*) (2011).
20. S. PREDA, V.S. TEODORESCU, A.M. MUSUC, C. ANDRONESCU, M. ZAHARESCU, Influence of the TiO_2 precursors on the thermal and structural stability of titanate-based nanotubes, *J. Mat. Res.*, 28, 294, 303 (2013).
21. A. VENTOSA, M.T. GARCIA, M. KAMEKURA, H. ONISHI, F. RUIZ-BERRAQUERO, *Bacillus halophilus* sp. nov., a new moderately halophilic *Bacillus* species, *Syst. Appl. Microbiol.*, 12, 162, 166 (1989).
22. R. COJOC, S. MERCIU, G. POPESCU, L. DUMITRU, M. KAMEKURA, M. ENACHE, Extracellular hydrolytic enzymes of halophilic bacteria isolated from a subterranean rock salt crystal, *Roum. Biotechnol. Lett.*, 14, 4658,4664 (2009).
23. M.A. HAYAT, Basic Electron Microscopy Technique. *Van Nostrand Reinhold Company, New York*. (1972).
24. E.S. REYNOLDS, The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *The J. Cell Biol.*, 17, 208, 212 (1963).
25. J. NADOR, N. ORGOVAN, M. FRIED, P. PETRIK, A. SULOK, J.J. RAMSDEN, L. KOROSI, R. HORVAT, Enhanced protein adsorption and cellular adhesion using transparent titanate nanotube thin films made by a simple and inexpensive room temperature process: application to optical biochips, *Colloids and Surfaces B: Biointerfaces*, 122, 491,497 (2014).
26. M. ENACHE, R. COJOC, M. KAMEKURA, Halophilic microorganisms and their biomolecules: approaching into frame of bio(nano)technologies, D. K. MAHESHWARI & M. SAEAF (eds.) *Halophiles – Biodiversity and sustainable exploitation*, Springer, Switzerland,161-172 (2015).