ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2014, *59*(2), 97-103

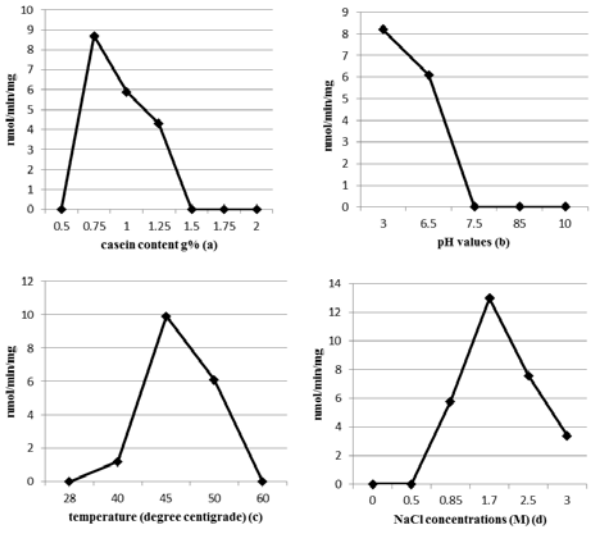
THE FUNCTIONALIZATION OF SILICA AND TITANATE NANOSTRUCTURES WITH HALOTOLERANT PROTEASES

Simona NEAGU,[a] Silviu PREDA,[b] Crina ANASTASESCU,[b] Maria ZAHARESCU,[b] Mădălin ENACHE[a,*] and Roxana COJOC[a]

[a] Institute of Biology Bucharest of the Roumanian Academy, 296 Splaiul Independenței, P.O. Box 56-53, Bucharest 060031, Roumania
[b] "Ilie Murgulescu" Institute of Physical-Chemistry of the Roumanian Academy, 202 Splaiul Independenței, Bucharest 060021, Roumania

Received November 6, 2013

Salted environments are the natural habitat of certain halophilic microorganisms, both bacteria and archaea extremophiles which are a source of enzymes (extremozymes) with extreme stability. Taking into account that extremozymes are stable and active under hard conditions of pH and ionic strength, their study as biocatalysts is attractive. Many halophiles secrete proteolytic enzymes which enable the degradation of proteins and peptides in the natural hypersaline environments. The interactions of various nanostructures with biological molecules attracted a serious interest in the last years in order to understand their use for human life purposes and benefits. This paper deals with approaching the functionalization of silica and titanate nanostructures with halotolerant proteases. Since salted environments are widely used in Roumania for recreational activity, it is of high interest to investigate the impact of new materials like silica and titanate nanotubes on metabolites of halophilic microorganisms that populated such areas. To the best of the author's knowledge, this is the first report on the investigation of the functionalization of silica and titanate nanostructures with halotolerant proteases of moderately halophilic bacterial strains isolated from salt massif dated from Neogene period.



INTRODUCTION

The interactions of various nanostructures with biological molecules attracted a serious interest in the last years in order to understand their use for human life purposes and benefits.[1] Nanostructures are made by several commonly used materials like metal oxides (titanium, zinc, iron). Such kinds of structures are characterized by chemical stability, electrical and magnetic properties, catalytic properties.[2,3] These unique features recommended the use of nanostructures in a wide spectrum of applications like antimicrobial agents, biosensor materials, cosmetic products, food and electronic components.[1,4,5] Over 200 market available products which use the term of "nanotechnology" are represented by solar protection glass, clothing with high resistance to dyes, sport equipment's and accessories and electronic devices.[6]

The biomolecules have been immobilized on polymeric matrix and inorganic support by various techniques based on physical adsorption,

* Corresponding author: madalin.enache@ibiol.ro; madalin_enache@yahoo.com

electrostatic bridges, specific affinity and covalent bridges.[3] Such kind of supports functionalized with proteins, enzymes, antibodies or DNA have been used in various biotechnological processes.[3] The adsorption of biomolecules on the nanoparticles surface has been made either for small size molecules like C vitamin,[7] or relatively high molecules like proteins and enzymes.[8,9]

It is well known today that salted environments are the natural habitat of certain halophilic microorganisms, both bacteria and archaea extremophiles which are a source of enzymes (extremozymes) with extreme stability. Taking into account that extremozymes are stable and active under hard conditions of pH and ionic strength, their study as biocatalysts is attractive. On the other hand, it is clear that some extremophiles could be considered as a novel source of enzymes with novel activities and applications.[10,11]

Many halophiles secrete proteolytic enzymes which enable the degradation of proteins and peptides in the natural hypersaline environments. These enzymes are serine proteases known as halolyzines and are likely to generate oligopeptide, dipeptide and amino acid intermediates which feed into central metabolisms. Little is known regarding the mechanisms used by haloarchaea to secrete extracellular proteases to the medium. General secretory (Sec) and twin-arginine-transport (Tat) pathways for protein secretion are conserved in the haloarchaea.[12,13]

The studies of microbial metabolites from biotechnological overview attracted researchers in all time periods. Although many haloarchaea secrete extracellular hydrolases (amylase, protease, lipase) only a few of them were investigated and characterized by researchers.[14] Proteases (intracellular and extracellular) participate in a number of biological processes such as the degradation of abnormal proteins, control of transcription factors, precursor processing, development and differentiation, regulation of cell cycle and apoptosis.[15,16] In addition, proteases have many practical applications in biotechnology. Between *Archaea,* halophilic microorganisms are the easiest to grow in laboratory and advanced genetic studies[17,18,19] argue for investigations of proteolysis and presence of novel proteases which can work as models for the study of the evolution and development of life in extreme conditions.

Since salted environments are widely used in Romania for recreational activity,[20,21] it is of high interest to investigate the impact of new materials like silica and titanate nanotubes on metabolites of halophilic microorganisms that populated such areas. To the best of the author's knowledge, this is the first report on the investigation of the functionalization of silica and titanate nanostructures with halotolerant proteases from moderately halophilic bacterial strains isolated from salt massif dated from Neogene period.

MATERIALS AND METHODS

Nano/microtubes preparation

The titanate nanotubes were prepared from titanium oxide by the hydrothermal method using various experimental conditions[22] and silica microtubes were obtained by sol-gel method using tetraethoxisilane as source of SiO_2 and racemic mixture of tartaric acid.[23] The experimental conditions used for both types of tubes are presented in the Table 1.

Nano/microtubes characterization

The structural characteristics of the synthesized nanostructures 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.

Table 1

Experimental conditions for titanate nanotubes and silica microtubes synthesis

Samples	Experimental conditions			
	Reaction mixture	Reaction time (h) / temperature (°C)	Time of post reaction thermal treatment (h)	Product characteristics
Silica	Tetraethoxisilan, as source of SiO_2 + racemic tartaric acid	24/20	5h 110 °C	Micrometric tubes, L≈2-5 μm Ø≈0.1-0.2 μm
Titanate	TiO_2 + NaOH	24/140	12 h 110 °C	Nanotubes, L≈ 50 nm, Ø ≈ 8-9 nm

Halobacterial strain used in the study and conditions for proteases obtaining

The moderately halophilic strain used in this study has been isolated from condense water accumulation in salt mine Unirea, from Slănic Prahova area in Roumania. The strain has been isolated on MH medium containing (g/L): yeast extract 10, proteose-peptone 5, glucose 1, NaCl 100, $MgCl_2 \times 6H_2O$ 7, $MgSO_4 \times 7H_2O$ 6, $CaCl_2 \times 2H_2O$ 0.36, KCl 2, $NaHCO_3$ 0.06, NaBr 0.026.[24] The preliminary characterization of the strain 2B39 related to the growth in the presence of chloramphenicol, sodium deoxycholate, the presence of extracellular hydrolytic activities, range of NaCl concentrations for growth and optimum NaCl concentration for growth was performed following the previously described protocols.[25]

The bacterial inoculum has been obtained on the MH culture medium and the biosynthesis parameters for strain 2B39 are represented by temperature of 28 °C, shaking at 300 rpm for 48 h at 1.7 M NaCl (10%). For proteases biosynthesis, the modified MH medium has been used with the following composition (g/L): yeast extract 10, NaCl 100, $MgCl_2 \times 6H_2O$ 7, $MgSO_4 \times 7H_2O$ 6, $CaCl_2 \times 2H_2O$ 0.36, KCl 2, $NaHCO_3$ 0.06, NaBr 0.026 and casein 10. The culture medium (100 mL) distributed in Erlenmayer flasks has been inoculated with 2 mL culture in exponential growth phase. After inoculation, the flasks were incubated for 48 h at 28 °C and 300 rpm shaking.

Isolation and partial purification of proteases

50 mL of microbial culture after 48 h of growth were centrifuged at 9500 rpm, 4 °C for 30 minutes. The supernatant has been treated with acetone 50% (cooled at 5 °C; 2:1 v/v) and centrifuged at 10000 rpm, 4 °C for 30 minutes. The resulted supernatant has been treated with acetone 80% and kept at 4 °C for 24 h. The resulted pellet has been obtained by centrifugation and dissolved in 20 mL buffer containing 20 mM Tris-HCl, pH 8, 0.5mM $CaCl_2$ and 0.1 M NaCl and dialyzed for 24 h under slight shaking conditions with the same buffer at 4 °C.

Determination of caseinolytic activity

The protein content has been determined using Lowry method. The caseinolytic activity has been determined following the method described by Kunitz and modified.[26] The sample containing 400 μL casein 0.5% (Merck) in 50 mM Tris-HCl buffer, pH 8 and 10% NaCl and 100μL enzyme extract was incubated on water-bath at 55 °C. After 30 minutes, the reaction was stopped by adding 500 μL of trichloroacetic acid. The samples were incubated at room temperature for 10 minutes, filtered and the absorbance at 280 nm has been determined using blank prepared with similar procedure but reaction stopped at time zero. The enzymatic activity was expressed as nmol/min/mg of protein.

Biochemical features of obtained proteases

The enzymatic extract obtained was investigated in order to reveal the influence of substrate concentration, pH values, ionic strength and temperature on the enzymatic activity. The optimum concentration of the substrate has been established varying casein content between 0.5-2% and maintaining the other parameters at optimum values. The influence of pH values on enzymatic activity has been determined on the substrate dissolved in buffer citric acid-Na_2HPO_4 0.1 M (pH 3-6.5), Tris-HCl 20 mM (pH 7-10). The optimum temperature for the enzymatic extract was evaluated by incubating the reaction mixture at several temperatures in range of 28-60 °C. The effect of ionic strength has been observed by varying NaCl concentrations from 0 until to 3.5 M.

Functionalization of proteases on silica and titanate nanostructures

In order to perform the immobilization 0.05 grams of titanate or silica nanostructures were added to seven milliliters of enzymatic extract, vigorously shake and kept at 4 °C for 16 hours. The exceeding volume of enzymatic extract was decanted and obtained pellet was washed with 10 mL of buffer containing 20 mM Tris-HCl, pH 8, 0.5 mM $CaCl_2$ and 0.1 M NaCl, and kept at 4 °C in 10 mL of the same buffer for further experiments. The protein content was determined by Lowry method in native enzymatic extract, in exceeding volume of enzymatic extract and in the buffer after washing procedure. In order to estimate the capacity of immobilization, the difference between initial amount of enzyme and amount from exceeding volume of enzymatic extract was calculated and correlated with one gram

of nanostructures. The capacity of immobilization was expressed as mg of enzyme/gram of nanostructures.

RESULTS AND DISCUSSION

Nanotubes features

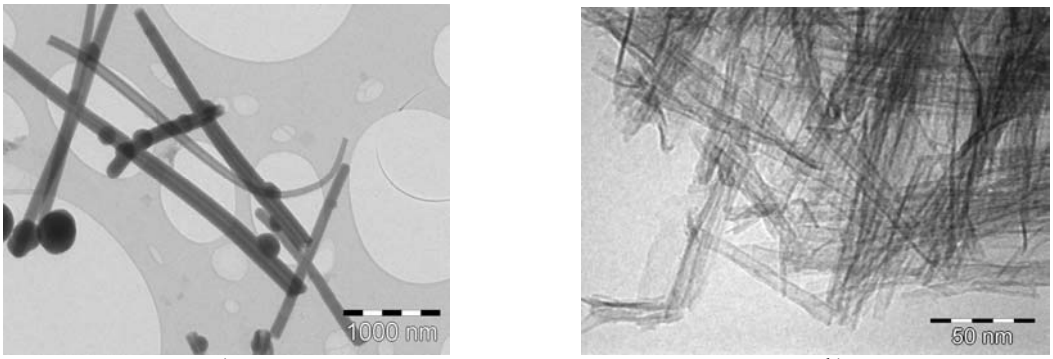
Various types of nanotubes were obtained by hydrothermal synthesis and microtubes by sol-gel synthesis under different conditions of temperature, reaction time and time of post reaction thermal treatment (Table 1). The morphological characteristics of the resulted nanotubes were assessed by electron microscopy (Fig. 1). The results showed that the titanate nanotubes are about 10 times smaller than the silica tubes. In the latter case besides tubes small amount of silica nanoparticles were formed.

In the same time silica tubes are amorphous,[23] while the titanate nanotubes have a sodium dititanate crystalline structure.[22] The inner diameter is of hundred of nanometers in the case of silica microtubes, but only few nanometers in the case of the titanate nanotubes.

Taking into consideration the differences concerning the composition of the two types of micro/nanotubes, but, especially, the differences in their chemical composition, structure and morphology it is expected to manifest different behavior to functionalization with halotolerant proteases.

Bacterial strain

We selected the halophilic bacterial strain 2B39 for further investigation. This bacterial strain was cultivable on medium supplemented with sodium deoxycholate, in the presence of high concentrations of NaCl, up to 3M with optimum at 1.7 M NaCl (Table 2) and was sensitive to chloramphenicol. The optical microscopically analysis showed that the investigated strain was represented by isolated bacilli, staining Gram-positive. The strain revealed the capacity to hydrolyze casein and Tween80, relatively small molecular weight substrates, but was unable to hydrolyze starch and carboxymethylcellulose which are polymeric compounds. According to this preliminary data, the strain appears to be a moderately halophilic bacterium, most probably to *Bacillus* sp.



a) b)

Fig. 1 – TEM image of the silica microtubes prepared by sol-gel method (a) and titanate nanotubes prepared by hydrothermal method (b) (Note: the magnification is in the case of titanate nanotubes 20 times higher).

Table 2

The range of NaCl for growth of the strain 2B39 and optimum concentration of NaCl for growth at 1.7 M

	0 M NaCl	1 M NaCl	1.7 M NaCl	2 M NaCl	3 M NaCl
24 hours	-	+	+	+/-	-
48 hours	-	+++	+++	++	-
72 hours	-	+++	+++	+++	++

Table 3

The biochemical parameters of purification steps of proteases produced by moderately halophilic bacterial strain 2B39

Purification steps	Volume (mL)	Protein content (mg/mL)	Enzymatic specific activity (nmol/min/mg)
Culture filtrate	50	2.75	60
Treatment with acetone 50%	45	1.62	74
Treatment with acetone 80%	90	0.95	73
Dialyzed proteic extract	17	0.40	16

Isolation and partial purification of proteases

Extracellular proteases obtained during to growth of halophilic bacterial strain 2B39 under temperature of 28 °C, shaking at 300 rpm for 48 h at 1.7 M NaCl (10%) has been purified according to upper described protocol. The general features of purification protocol are summarized in Table 3. Generally, the specific enzymatic activity increases in each step of the purification procedure. In the last step of the protocol, most probably due to the buffer low content in NaCl, the specific activity is modified if compared with the previous steps (Table 3). In this way, the obtained results revealed that enzymatic activity of dialyzed proteic extract appears to be lower than the activity of native proteic extract. These data could suggest that enzymatic activity has been partially lost into the dialysis steps, even if the calcium ions were present in used buffers. It is well known that the calcium ions are acting as proteases stabilizers,[15] being out of the prostatic group of the enzyme. According to the review literature data,[15] the calcium ions stimulate the proteases activity by promoting protein conformation highly active from catalytic point of view. During our experiments, most probably due to the low content in NaCl of the dialysis buffer, the proteases could lose the catalytic activity (Table 3).

According to the data revealed by purification step after treatment with acetone 50% (2:1 v/v), the further experiments for the immobilization of proteases on the titanate and silica nanostructures were conducted with enzyme extract obtained in this step.

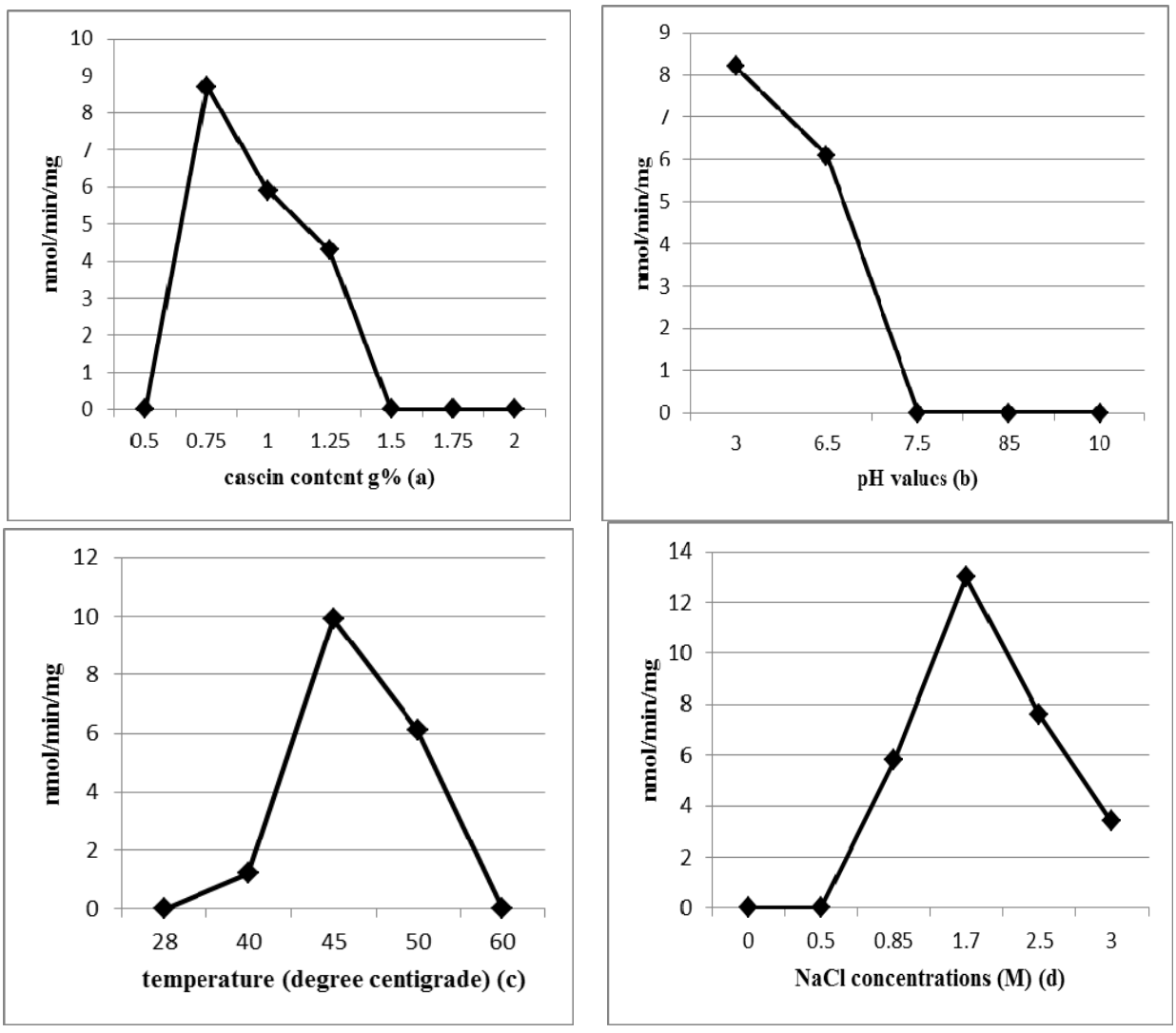


Fig. 2 – Biochemical features of investigated proteases: substrate (casein) concentration (a); pH values (b); temperature (c) and ionic strength (NaCl concentrations) (d).

Table 4

The immobilization of the proteases on the silica/titanate nanostructures; 1 = native enzyme solution; 2 = exceeding volume of enzymatic extract; 3 = buffer after washing procedure

Silica/***Titanate***	Protein content (mg/mL)			Immobilization capacity (mg/g)	Specific activity (nmol/min/mg)		
	1	2	3		1	2	3
Acetone treatment 50%	2.1/***2.1***	1.8/***1.6***	0.1/***0.2***	2.7/***7.5***	70/***68***	68/***65***	6/***4***
Dialyzed extract	0.4/***0.5***	0.3/***0.2***	0.1/***0.08***	1.8/***4.5***	6/***4***	5/***2***	0.5/***0***

Biochemical features of obtained proteases

Related to biochemical features of the investigated proteases, according to the data showed in Fig. 2 appears that optimal activity is recorded in the presence of 0.75% of substrate (casein) (Fig. 2a), at pH value of 3 (Fig. 2b), 45 °C (Fig. 2c) and in the presence of 1.7 M NaCl in the reaction mixture (Fig. 2d). These parameters revealed a behavior for enzyme capable of degrading macromolecular compounds at low concentrations and the adsorption of the enzyme to the substrate during to catalytic pathway of hydrolysis. The biochemical features of the investigated enzyme revealed also a first degree kinetic of Michaelis-Menten parameters and could be considered as a support for adaptation of bacterial strain to the ionic strength of salted environments taking into account that similar bacterial strains are able to degrade strong polluting chlorinated pesticides in saline environments following similar kinetic behavior.[27]

Functionalization of proteases on silica and titanate nanostructures

The obtained data revealed that titanate nanotubes showed a high immobilization capacity if compared with silica microtubes and this capacity appears to be three time higher (Table 4). This capacity is not affected by the purification steps of the enzyme, being maintained either for the partially purified enzyme by acetone treatment or the dialyzed enzyme. This higher capacity of immobilization of the titanate nanotubes could be assigned to the differences of their composition, structure and morphology as compared to the silica nanotubes, as it was mentioned above. Specific surface area, as determined by BET for the titanate nanotubes, was 321.44 m²/g and the total pore volume of pores was 0.85 cm³/g. These high values and the local chemical interactions explain the capacity of immobilization.

The obtained results revealed that enzymatic activity appears to be relatively similar when partially purified enzyme is used either for titanate or silica nanostructures. In the case of the purified enzyme, the catalytic activity appears to double when the immobilization was done on silica microtubes, even if this substrate didn't show a high capacity of enzyme immobilization (Table 4).

CONCLUSIONS

The capacity of immobilization of the proteolytic enzymes is higher on the titanate nanotubes (around three times) if compared with silica microtubes, even if for the immobilization has been used partially purified enzyme or dialyzed enzyme (Table 4). This higher capacity of immobilization of the titanate nanotubes could be assigned to the differences of their composition, structure and morphology as compared to the silica nanotubes, as it was mentioned above. The enzymatic activity appears similar when using for immobilization partially purified enzyme. The activity is higher on the immobilization on the silica nanostructures even if this substrate revealed a low capacity of enzyme adsorption via electrostatic behaviors.

REFERENCES

1. H. Kaur, S. Kaur and M. Singh, *Biologia*, **2013**, *68*, 1048-1053.
2. E. Katz, A. N. Shipway and I. Willner, in "Nanoparticles – From Theory to Applications", G. Schmidt (Ed.), Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2003, p. 368-421.
3. E. Katz and I. Willner, *Angew. Chem. Int. Ed.*, **2004**, *43*, 6042-6108.
4. P. Mukherjee, A. Ahmad, D. Mandal, S. Senapati, R. S. Sainkar, I. M. Khan, R. Parishcha, V. P. Ajaykumar, M. Alam, R. Kumar and M. Sastry, *Nano Lett.*, **2001**, *1*, 515-519.
5. Sondi and I. Salopek-Sondi, *J. Colloid Interface Sci.*, **2004**, *275*, 177-182.

6. www.nanotechproject.org/index.php?id=44
7. T. Rajh, J. M. Nedeljkovic, L. X. Chen, O. Poluektov and M. C. Thurnauer, *J. Phys. Chem. B*, **1999**, *103*, 3515-3519.
8. G. T. Hermanson, A. K. Mallia and P. K. Smith, "Immobilized Affinity Ligand Techniques", 1st ed., Academic Press Limited, London, 1992.
9. S. V. Rao, K. W. Anderson and L. G. Bachas, *Microchim. Acta,* **1998**, *128*, 127-143.
10. Oren, *J. Ind. Microbiol. Biotechnol.*, **2002**, *28*, 56-63.
11. M. Enache, G. Popescu, T. Itoh and M. Kamekura, in "Adaption of Microbial Life to Environmental Extremes", H. Stan-Lotter and S. Fendrihan (Eds.), Springer Wien New York, Vienna, 2012, p. 173-197.
12. G. Ring and J. Eichler, *J. Bioenerg. Biomembr.*, **2004**, *36*, 35-45.
13. M. Pohlschröder, E. Hartmann, N. J. Hand, K. Dilks and A. Haddad, *Ann. Rev. Microbiol.*, **2005**, *59*, 91-111.
14. M. Enache and M. Kamekura, *Rom. J. Biochem.*, **2010**, *47*, 47-59.
15. R.E. De Castro, J. A. Maupin-Furlow, M. I. Gimenez, M. K. Herrera Seitz and J. J. Sanchez, *FEMS Microbiol. Rev.*, **2006**, *30*, 17-35.
16. W. Shi, X. F. Tang, Y. Huang, F. Gan, B. Tang and P. Shen, *Extremophiles*, **2006**, *10*, 599-606.
17. T. Allers and M. Mevarech, *Nat. Rev. Genet.*, **2005**, *6*, 58-73.
18. S. DasSarma, B. R. Berquist, J. A. Coker, P. DasSarma and J. A. Müller, *Saline Systems***, 2006**, *2*, Art. 3 (12 pages).
19. S. DasSarma, *Microbe*, **2006**, *1*, 120-126.
20. V. A. C. Bulgăreanu, *Lakes & Reservoirs: Research & Management*, **1996**, *2*, 211-229.
21. M. Enache, in "Complexitatea biologică sub aspect macro, micro, nano" (in Romanian), M. Enache (Ed.), Publishing House of the Romanian Academy, Bucharest, 2011, p. 117-126.
22. S. Preda, V. S. Teodorescu, A. M. Muşuc, C. Andronescu and M. Zaharescu, *J. Mater. Res.*, **2013**, *28*, 294-303.
23. C. Anastasescu, M. Anastasescu, V. S. Teodorescu, M. Gartner and M. Zaharescu, *J. Non-cryst. Solids*, **2010**, *356*, 2634-2640.
24. A. Ventosa, M. T. Garcia, M. Kamekura, H. Onishi and F. Ruiz-Berraquero, *Syst. Appl. Microbiol.*, **1989**, *12*, 162-166.
25. R. Cojoc, S. Merciu, G. Popescu, L. Dumitru, M. Kamekura and M. Enache, *Rom. Biotechnol. Lett.*, **2009**, *14*, 4658-4664.
26. C. Sanchez-Porro, S. Martins, E. Mellado and A. Ventosa, *J. Appl. Microbiol.*, **2003**, *94*, 295-300.
27. T. Oncescu, P. Oancea, M. Enache, G. Popescu, L. Dumitru and M. Kamekura, *Cent. Eur. J Biol.*, **2007**, *2*, 563-573.