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PRELIMINARY DATA CONCERNING COMMUNITIES OF MICROORGANISMS IN A VOLCANIC TUFF ENDOLYTIC HABITAT

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Abstract. The current study revealed the presence of biofilms of some bacterial communities on the surface of some volcanic tuff from Teişani and Malul Alb. A relatively high number of colony-forming units (CFU), respectively $7x10^4$ in the case of Teişani sample and $1.5x10^5$ in the case of Malul Alb sample, per gram of rock, have been isolated. Except for the presence of coliform bacteria in the Malul Alb rock in number of 1.4x10 CFU, other categories of microorganisms detected both in Teişani and the Malul Alb rock (volcanic tuff) were represented by ammonifying, denitrifying, and sulphate reducing bacteria. In terms of chemical composition, the two types of volcanic tuffs are similar, with only a slight difference in the percentage of potassium and calcium.

Keywords: microbial communities, rock biofilms, volcanic tuff, Teişani, Malul Alb.

Rezumat. Date preliminare privind comunitățile de microorganisme din habitate endolitice de tip tuf vulcanic. Prezentul studiu a relevat prezența sub forma de biofilme a unor comunități bacteriene pe suprafața unor tufuri vulcanice din zonele Teișani si Malul Alb. S-a izolat un număr relativ mare de unități formatoare de colonii (UFC), respectiv $7x10^4$ în cazul probei Teișani și $1,5x10^5$ în cazul probei Malul Alb, per gram de rocă. Cu excepția prezenței bacteriilor coliforme în număr de 1,4x10 UFC în roca de la Malul Alb, alte categorii de microorganisme detectate atât în roca de la Teișani, cât și în roca de la Malul Alb (tuf vulcanic) au fost reprezentate de bacterii amonificatoare, denitrificatoare și sulfat reductoare. În privința compoziției chimice cele două tipuri de tuf vulcanic sunt similare, între ele fiind o uşoară diferenţă în procentul de potasiu şi calciu.

Cuvinte cheie: comunități microbiene, tuf vulcanic, Teișani, Malul Alb.

INTRODUCTION

Due to their metabolic versatility, microorganisms have a major impact on various life activities like human health, environmental management, crop growth, agriculture, industrial and food biotechnologies, and bioprocessing. The studies of microorganisms over years conducted to accumulate a lot of data and modern approaches like machine–learning methods have been developed in their investigation (QU et al., 2019). Over the years, the limited conditions under which life forms can be found have been investigated in many directions, including wide range of physicochemical factors such as temperature, pH, pressure, radiation, salinity, and nutrient availability. Thus, extremophilic microorganisms are adapted to these conditions and have developed metabolic and biochemical pathways for growth and development. Many areas of our planet host optimal development conditions for such microorganisms, sometimes more physicochemical parameters providing poli-extreme conditions (MERINO et al., 2019).

In this context, it can be considered that polyextremophilic microorganisms are the most abundant life forms on Earth. Studies conducted over the years have led to the isolation of cultivable polyextremophilic microorganisms but also the identification of non-cultivable microbial communities through various independent approaches to cultivation and which have provided new data on the limits of life (SYSOEV et al., 2021). Thus, research on extremophilic microorganisms has brought real advantages to molecular biology and medicine. Data from the literature describe in detail the extremophilic microorganisms including their phylogeny and genetics (DASSARMA et al., 2019).

The endolytic environments represented mainly by the porous spaces of the rocks are colonized by different categories of microorganisms such as cosmoendolytic, cryptoendolytic, and endolytic. The first category is found in holes, cracks, and fissures in the rock, the second in the porous spaces of the mineral, and the last in the pores resulting from the latent metabolic activity of some microorganisms. Other types of endolytic habitats host epilithic and hypolytic microorganisms (GOUBLIC et al., 1981; COCKELL et al., 2005; JEFFREY et al., 2007; OMELON CR, 2016).

Enzymes of polyextremophilic microorganisms are of particular interest because they are active in natural environments with low water activity. Between them, the proteases represent a wide segment of the industrial market of detergents, disinfectants, food processing, tanneries, and biocatalysts in organic syntheses but also in the medical field (BENMEBARECK et al., 2020; DAS SARMA et al., 2019). In the present paper we studied the volcanic tuffs from the Teişani area, located near the salt deposit from Slănic Prahova, and the one from the Malul Alb area located near the salt plateau from Meledic in Buzau County.

The present study aimed at the isolation, purification, and identification of endolytic microorganisms from the investigated areas, their involvement in biogeochemical circuits, and their characterization in terms of extracellular hydrolytic enzyme spectrum. According to the authors knowledge, this is the first biological and microbiological investigation on volcanic tuff rock from Malul Alb.

MATERIALS AND METHODS

Investigated areas and sampling collection

The analysed samples were fragments of volcanic tuff rock taken from the Teişani and Malul Alb areas. The volcanic tuff rock from Teişani area is located near the village of the same name in Prahova County and near the salt deposit from Slănic Prahova and the volcanic tuff from Malul Alb is located near the Meledic salt plateau in Buzau County. Samples were represented by rock fragments of several dimensions and weight that were kept after sampling in appropriate conditions during transportation to the laboratory.

Culture media and growth condition

For quantitative determination of the number of heterotrophs, coliforms, streptococci, sulphate reducing, ammonifying and denitrifying bacteria, one gram of rock sample was milled and then suspended in 10 mL of physiological serum. This solution was further used for decimal dilution preparation in all experimental cases. The culture media has been solidified by adding 20 g/L agar. The media composition is detailed in Table 1 and the experiment has been conducted as described in previous papers (COJOC et al., 2013; LUCACI et al., 2019; RUGINESCU et al., 2022).

Table 1. The culture medium used in the current experiments related to this paper.

Postgate medium (g/L)	Levine medium (g/L)	Sodium azide medium (g/L)
NH_4Cl – 1 K_2HPO_4 – 0.5 $MgSO_4$ – 2 Na_2SO_4 – 0.5 Calcium lactate – 3.5 Distilled water – 1 L Sterilization at 120°C for 30 min Adding to 500 mL solution: 5% Yeast extract – 10 mL 5% $FeSO_4$ – 5 mL 1% Na_2S – 2 mL 10% $NaHCO_3$ until to reach pH 7.2 – 7.4	Peptone – 10 Lactose – 10 K_2HPO_4 – 2 Eosin Y – 0.4 Methylene blue – 0.065 Agar – 15 Distilled water – 1 L	Proteose peptone – 20 Glucose – 5 NaCl – 5 K_2HPO_4 – 2.7 KH_2PO_4 – 2.7 Sodium azide – 0.4 Ethanolic solution 1.6% of bromcresol purple – 2 mL Distilled water – 1 L
pH – 7 – 7.2; autoclaving 120°C for 30 min.	pH – 7 – 7.2; autoclaving 120°C for 30 min.	pH – 7 – 7.2; autoclaving 120°C for 30 min.
Sulphate reducing bacteria	Coliform bacteria	Streptococcus bacteria

Nutrient broth medium (g/L)	Ammonifying medium (g/L)	Pochon medium (g/L)
Peptone – 10 Beef extract – 10 NaCl – 5 Distilled water – 1 L	Standard saline solution Winogradski – 50 mL Asparagine – 0.2 Oligoelement solution Wan Niell – 1 mL Distilled water – 1 L	Standard saline solution Winogradski – 50 mL KNO_3 – 2 Glucose – 10 $CaCO_3$ – 5 Distilled water – 1 L
pH – 7 – 7.2; autoclaving 120°C for 30 min.	pH – 7 – 7.2; autoclaving 120°C for 30 min.	pH – 7 – 7.2; autoclaving 110°C for 30 min.
Heterotrophic bacteria	Ammonifying bacteria	Denitrifying bacteria

Optical microscopy investigation – has been performed using a Zeiss Axio Imager.M2m microscope equipped with a camera photo in order to register the data.

XRF investigation – was performed by X-ray fluorescence using an XRF Rigaku ZSX100e Supermini system (Rigaku, Japan), following the manufacturer's protocol. After grinding with a pestle, the chemical composition of powdered material was determined and reported as a mass percentage (%).

SEM – The experiments for scanning electron microscopy involved high vacuum conditions. In order to be investigated, milled rock samples have been pre-treated by dispersion in 30% ethanol solution, and 10 µl of suspension were deposited on the microscope slide and sputtered with a gold layer. After the evaporation of ethanol, the prepared slides were dried in a vacuum followed by metal coating sputter. The used instruments are Jeol JSM-6610LV and coater JFC-1300.

RESULTS AND DISCUSSIONS

The general overview of investigated areas can be observed in Fig. 1. Both sampling sites appear as green, slightly blue colour rock, apparently without any form of life. However, in the proximity, the versants are rich in vegetation meaning that some debris from those areas may be rain transported to inhabit the sampling site. Considering this, we have in view by, several sampling campaigns, to separate allochthonous invasive microbiota from the native one. The investigation results presented in this study refer to some bacterial strains isolated from the rock samples taken after a relatively long dry season with missing precipitation, in order to have a preliminary overview on native rock microbiota.

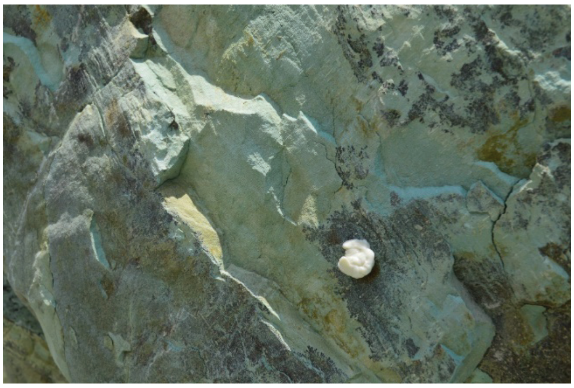


Figure 1. General overview of the investigated volcanic tuff, left – Teişani, right – Malul Alb (original).

The chemical composition of the investigated samples (Table 2) was determined using X-ray fluorescence spectrometry using one gram of each sample, the analysis being carried out in a helium atmosphere according to a protocol recommended by the manufacturer.

The results show that, regarding the sample from Malul Alb, 12 elements predominated, six of which, namely Si, Al, Ca, K, Fe, and Ti, had a concentration greater than 1% of the mass of the analysed sample (Table 2). A series of other elements from the lanthanides and actinides category were detected in traces.

Regarding the sample from Teişani, the same 12 elements as at Malul Alb were predominant; in this case, the percentage of calcium was higher than that of potassium, while the percentage of potassium was higher at Malul Alb. Similarly, traces of lanthanides and actinides were detected. The chemical composition showed a high similarity between the two categories of samples investigated in this study. Data from the literature (MOCANU et al. 2008) show a similar chemical composition obtained by EDAX analysis for the volcanic tuff from Teişani. The authors of the study identified similar chemical elements to those of the sample analysed in our present study, respectively Al, Si, K, Ca, and Fe, and the mass percentages obtained were relatively similar, except for silicon.

Table 2. Chemical composition of the investigated rock samples.

Chemical elements	%mass	
	Teişani - Prahova	**Malul Alb - Buzau**
SiO_2	77	72
Al_2O_3	8	10
Cao	4,16	6,8
K_2O	4,49	4,67
Fe_2O_3	2,44	3,82
TiO_2	1,26	1,22
Traces of:	P_2O_5, S_2O_3, Ag_2O, MnO, SrO, lanthanides and actinides	

A relatively high number of colony-forming units (CFU) has been recorded for the rock investigated samples, comparatively with the number characteristic for lakes and other similar environments or rock salt (ENACHE et al., 2008; COJOC et al, 2009). Comparatively, the Dead Sea and Great Salt Lake are inhabited by halophilic microorganisms of the magnitude of 10^6 cells/g, although fluctuating periodically (POST, 1977; OREN, 1993) and Lake Chaka in north-western China by 4.8 x 10^6 cells/ml (JIANG et al., 2006) and other rock are the host for 10^3 CFU per gram of stone (KRUMBEIN, 1969; ECKHARDT, 1985).

As shown in Table 3, the number of CFU per gram of rock was $7x10^4$ for the Teişani sample and $1.5x10^5$ for the Malul Alb sample. Except for the presence of coliform bacteria in Malul Alb rock in number of 1.4x10 CFU, other categories of microorganisms detected both in Teişani and Malul Alb rock (volcanic tuff) were represented by ammonifying, denitrifying and sulphate reducing bacteria in variable CFU number as detailed in Table 3.

Both investigated volcanic tuff samples were not inhabited by the nitrifying bacteria, faecal streptococcus and coliform bacteria. The very well-developed microbial community on the surface of the investigated volcanic tuff rock are supported by the data from Fig. 3 that shows the development of biofilms both in the case of Teişani and Malul Alb samples. Similar data were also reported in literature regarding the biofilms on tuff stones at historical sites (CENNAMO et al., 2013).

It is relatively well known that characterization and taxonomical approach of biofilm forming microorganisms involve optical and electron microscopy techniques (TURNER & RICHARDS, 1984).

In this context, the results of Fig. 3 indicate that the analysed rock samples are covered by a community of microorganisms that have formed biofilms, despite the fact that some ecological constraints may limit its growth (KIEFT et al., 1996). This community has microorganisms engaged in the biogeochemical cycles of carbon, nitrogen, and sulphur (Table 3), as well as the metabolic capacity to utilize resources and cycle them with maximum energetic efficiency. On the other hand, such ability of microbial communities to develop and form biofilms on the stone surface has a negative impact on buildings and art object. Concerning this, several techniques for disrupting biofilms have been developed (CENNAMO et al., 2013).

Table 3. The number of cultivable microorganisms isolated from investigated rock samples.

Bacteria group	Teişani - Prahova	Malul Alb - Buzau
Coliforms bacteria (CFU/mL)	0	1.4x10
Heterotrophic bacteria (CFU/mL)	$7x10^4$	$1.5x10^5$
Ammonifying bacteria (cells/mL)	$3x10^4$	$1.4x10^3$
Denitrifying bacteria (cells/mL)	$4x10^3$	3x10
Sulphate reducing bacteria (cells/mL)	0.4	$5.5x10^2$

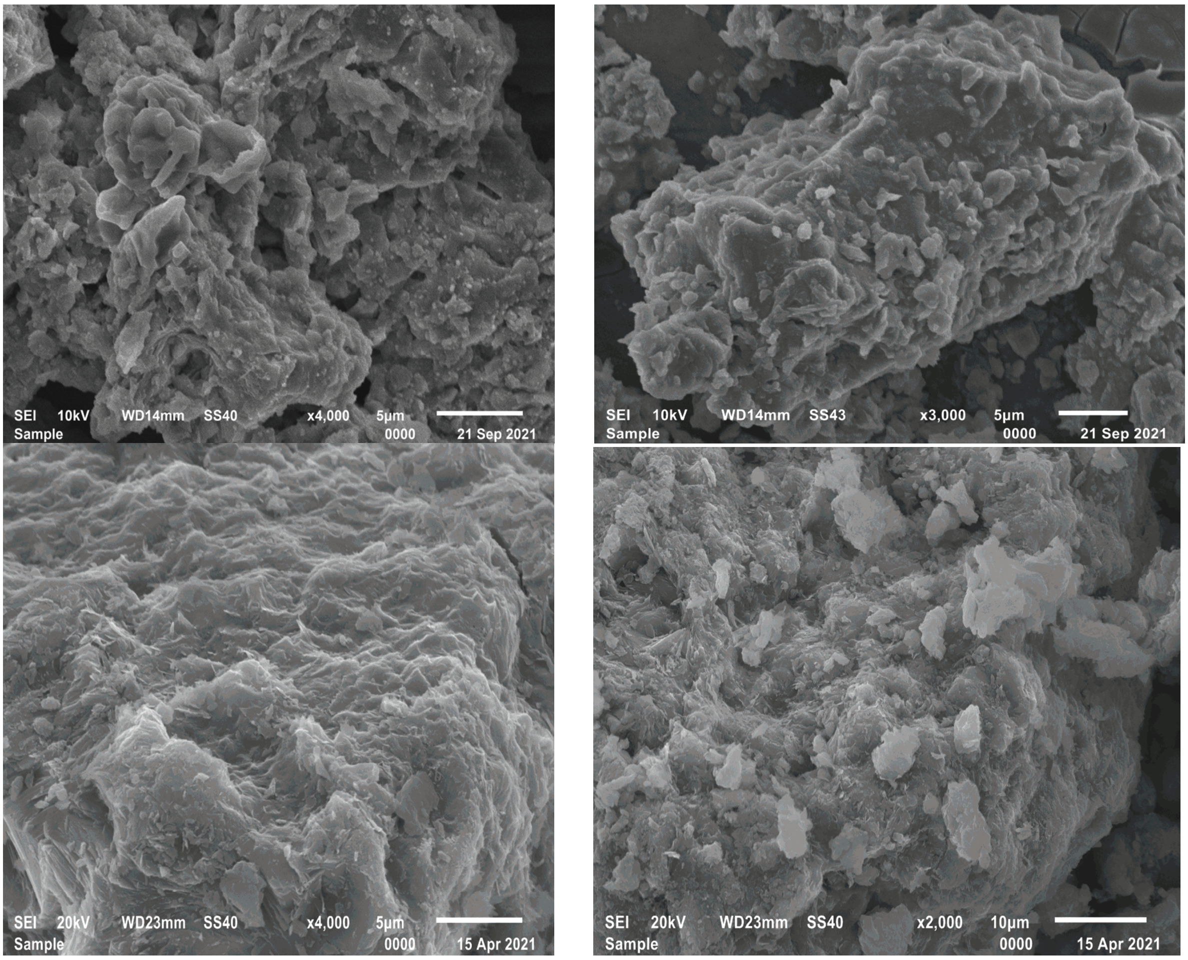

Figure 3. Biofilms observed by SEM investigation of the volcanic tuff, upper – Teişani, bottom – Malul Alb.

From this relatively high number of CFU for heterotrophic bacteria, 32 strains from the volcanic tuff of Teişani and 13 from Malul Alb have been randomly selected. These strains were characterized in terms of morphology and some biochemical features for the future purpose of molecular identification. Thus, as revealed by the data from Fig. 4, the predominant morphology of the selected strains in this study was rod and cocci.

They were either Gram-negative or Gram-positive, singular or associated forms. In the case of the samples from Teişani, 22 strains were Gram-positive and 10 Gram-negative, 29 had positive catalase and oxidase activity. In the case of the samples from Malul Alb, 10 had positive catalase activity and 4 had positive oxidase activity.

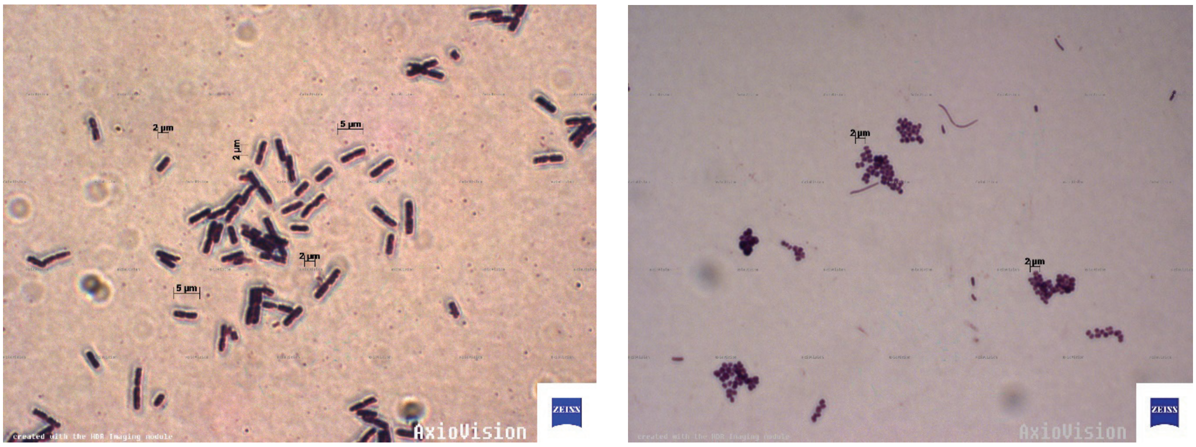

Figure 4. Morphology of some cultivable strains isolated from Teişani (left) and Malul Alb (right).

CONCLUSIONS

It can be stated that, from a chemical point of view, the two types of volcanic tuffs are similar, with only a slight difference in the percentage of potassium and calcium. Streptococci and faecal coliforms, nitrifying bacteria, were not identified in both samples. In the case of the sample from Teişani, no coliform bacteria were identified. Heterotrophic bacteria were identified in greater numbers in Malul Alb and less in Teişani, ammonifiers in greater numbers in Teişani and less in Malul Alb, denitrifiers in greater numbers in Teişani and less in Malul Alb, sulphate-reducing bacteria in high number at Malul Alb. In addition, coliform bacteria were identified at Malul Alb.

Regarding the activity of catalase and oxidase, the three strains with a negative response to the tests, in the case of the samples from Teişani, were different, while at Malul Alb they were the same. The investigated areas are characterized by the presence of a significant microbial community that mainly develops under biofilm form.

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REFERENCES

BENMEBAREK HANIA, ESCUDER-RODRIGUEZ J. J., GONZALEZ-SISO MARIA-ISABEL, KARROUB KARIMA. 2020. Test for the production and assay of the proteolytic activities of halophilic bacteria and archaea isolated from Algerian hypersaline environments. *Proceedings – MDPI*. University Press. London. **66**(1): 12.

COCKELL C. S., LEE P., BROADY P., LIM S. S. DARLENE, OSINSKI G. R., PARNELL J., KOEBERL C., PESONEN L., SALMINEN JOHANNA. 2005. Effects of asteroid and comet impacts on habitats for lithophytic organisms – A synthesis. *Meteoritics & Planetary Science*. Wiley-Blackwell Publisher. London. **40**(12): 1901-1914.

COJOC ROXANA, MERCIU SIMONA, POPESCU G., DUMITRU L., KAMEKURA M., ENACHE M. 2009. Extracellular hydrolytic enzymes of halophilic bacteria isolated from a subterranean rock salt crystal. *Romanian Biotechnological Letters*. University of Bucharest Press. **14**(5): 4658-4665.

COJOC ROXANA, ENACHE M., CODREANU-BĂLCESCU DOINA. 2013. Approaching the halophilic bacteria presence as possible indicator of biological dynamics in hypersaline aquatic environments. *DROBETA, Seria Ştiinţele Naturii*. Muzeul Regiunii Porţilor de Fier. **23**: 40-50.

DASSARMA P., COKER J. A., HUSE VALERIE, DASSARMA S. 2010. *Halophiles, Industrial Applications*. In: Flickinger M. C. (ed) Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology. John Wiley & Sons Inc. 9 pp.

ECKHARDT F. E. W. 1985. *Solubilization, Transport, and Deposition of Mineral Cations by Microorganisms - Efficient Rock Weathering Agents*. In: Drever, J. I. (eds) The Chemistry of Weathering. Nato ASI Series. Springer, Dordrecht. **149**. 23 pp.

ENACHE M., ITOH T., KAMEKURA M., POPESCU GABRIELA, DUMITRU LUCIA. 2008. Halophilic archaea isolated from man-made young (200 years) salt lakes in Slănic, Prahova, Romania. *Central Eurasian Journal Biology*. Springer. Berlin. **3**: 388-395.

GOUBLIC S., FRIEDMANN I., SCHNEIDER J. 1981. The lithobiontic ecological niche, with special reference to microorganisms. *Journal of Sedimentary Research*. Elsevier. Paris. **51**: 475-478.

JEFFREY W. J. & PACE N. R. 2007. Endolithic microbial ecosystems. *Annual Review Microbiology*. Oxford University Press. London. **61**: 331-347.

JIANG H., DONG H., ZHANG G., YU B., CHAPMAN R. LEAH, FIELDS M. W. 2006. Microbial diversity in water and sediment of Lake Chaka an athalassohaline lake in northwestern China. *Applied Environmental Microbiology*. American Society for Microbiology Publisher. New York. **72**(6): 3832-3845.

KIEFT T. L., KOVACIK W. P., TAYLOR JENNIFER. 1996. Factors limiting microbial activity in volcanic tuff at Yucca Mountain. *Department of Energy of United States*. Washington. N. p. Web doi: 10.2172/379091 (accessed February, 2022).

KRUMBEIN W. E. 1969. Über den Einfluß der Gesteinverwitterung auf die exogene Dynamik. *Geol. Rundsch*. Springer. Berlin. **58**: 333-363.

LUCACI ANCA IOANA, NEAGU SIMONA, COJOC ROXANA, RUGINESCU R., ARDELEAN I., ENACHE M. 2021. Benefits of understanding the enzymatic activities in saline Lake Letea in the Danube Delta. *Romanian biotechnological letters*. Bucharest. **26**(2): 2448-2454.

MERINO NANCY, ARONSON S. HEIDI, BOJANOVA P. DIANA, FEYHL-BUSKA J., WONG M. L., ZHANG S., GIOVANNELLI D. 2019. Living at extremes: Extremophiles and the limits of life in a planetary context. *Frontiers in microbiology*. Springer. Berlin. **10**: 780.

MOCANU B. I., NAUM N., LUNGU C., BOMBOS D., BOMBOS MIHAELA. 2008. Compositional characteristics of Piatra - Verde Slănic zeolitic vulcanic tuff. *Revista de Chimie*. București. **59**(7): 730-733.

OMELON C.R. 2016. Endolithic microorganisms and their habitats. In: Hurst C. J. (ed), *Their world: a diversity of microbial environments*. *Advances in environmental microbiology*. Springer, Cham. **1**: 171-201.

OREN A. 1993. The Dead Sea - alive again. *Experientia*. Springer. Berlin. **49**: 518-522.

POST F. J. 1977. The microbial ecology of the Great Salt Lake. *Microbial Ecology*. Springer. Berlin. **3**: 143-165.

QU K., GUO FEI, LIU X., LIN Y., ZOU Q. 2019. Application of Machine Learning in Microbiology. *Frontiers Microbiology*. Springer. Berlin: 10-827.

RICHARDS S. R. & TURNER R. J. 1984. A comparative study of techniques for the examination of biofilms by scanning electron microscopy. *Water Research*. Elsevier. Paris. **18**(6): 767-773.

RUGINESCU R., ENACHE M., POPESCU O., GOMOIU IOANA, COJOC ROXANA, BATRINESCU-MOTEAU C., MARIA G., DUMBRAVICIAN M., NEAGU S. 2022. Characterization of Some Salt-Tolerant Bacterial Hydrolases with Potential Utility in Cultural Heritage Bio-Cleaning. *Microorganisms*. MDPI Press. London. **10**(3): 644.

SYSOEV M., GRÖTZINGER S. W., RENN D., EPPINGER J., RUEPING M., KARAN R. 2021. Bioprospecting of Novel Extremozymes From Prokaryotes - The advent of Culture - Independent Methods. *Frontiers Microbiology*. Springer. Berlin. **12**: 630013.

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