SUPEROXIDE DISMUTASE ACTIVITY IN MICROORGANISMS INHABITING VOLCANIC TUFF ROCK

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Abstract. In this study, a number of 64 strains of microorganisms isolated from volcanic tuff rock from the Teișani (Prahova) and Malul Alb (Buzau) areas were tested for superoxide dismutase (SOD) activity. The protein concentration ranged from 0.35 in the P.A.-3 strain isolated from the rock in the Malul Alb area to 7.18 mg/mL in the case of rainwater leached from the slope in the Teișani area. SOD activity fell within the range of 136.7637 U/mg (sample T3-5) and 6628.25 U/mg (sample P.A.-3). Both SOD and alkaline phosphatase activities fluctuate, showing a mechanism of adaptation to the environmental conditions on the surface of the volcanic tuff rock.

Keywords: Superoxide dismutase, alkaline phosphatase, volcanic tuff rock.

Rezumat. Activitatea superoxid dismutazei la microorganisme care se dezvoltă în roca de tuf vulcanic. În cadrul acestui studiu un număr de 64 de tulpini de microorganisme izolate din roca de tuf vulcanic provenită din zonele Teișani (Prahova) și Malul Alb (Buzău) au fost testate pentru activitatea superoxid dismutazei (SOD). Concentrația de proteină a variat între 0,35 la tulpina P.A.-3 izolată din roca din zona Malul Alb și 7,18 mg/mL în cazul apei de ploaie levigate de pe versantul din zona Teișani. Activitatea SOD s-a încadrat în intervalul 136,7637 U/mg (proba T3-5) si 6628,25 U/mg (Proba P.A.-3). Atât activitatea SOD cât și cea a fosfatazei alcaline fluctuează arătând un mecanism de adaptare la condițiile de mediu de la suprafața rocii de tuf vulcanic.

Cuvinte cheie: Superoxid dismutaza, fosfataza alcalină, roca de tuf vulcanic.

INTRODUCTION

The diversity of endolytic microorganisms in the biosphere and their abundance demonstrate their ability to transform energy from various substrates as well as the capacity to inhabit a wide range of natural conditions (GOLUBIC et al., 1981). Moreover, the complexity of microbial communities is correlated with the conditions offered by the habitat for its colonization and the availability for energy and carbon sources. In various cases, habitats have extreme climatic or environmental conditions, thus limiting the number of species that may exist in such an ecological niche (OMELON, 2016). An example of such natural selection can be found in endolytic habitats which are colonized by microorganisms involved in their biodegradation or biodeterioration (ZHANG et al., 2019). Except for the anthropogenic impact, such habitats can be shaped by a series of climatic factors or by the impact with other external objects that can influence the porosity and cracks of the rocks, thus favoring the development of microorganisms. Almost all types of lithic habitats (chasmoendolithic, cryptoendolithic, euendolithic, hypolithic, hypoendolithic) and the microbial communities that populate them are subject to such natural modeling (COCKELL et al., 2005).

Widely distributed on the world, extremely and hostile habitats have the ability to harbor a huge diversity of both bacterial and archaeal species. Within this high diversity (chemically, biologically or physico-chemically) a lot of inhabitants have the ability and capacity to develop both particular survival strategies or to synthesize biologically active compounds under bio-physiologically or chemically environmental stress conditions.

Microorganisms have the ability to colonize buildings and architectural monuments or stone sculptures at different times of their existence (ZHANG et al., 2019). This causes them to be intensively investigated through different methods to identify mechanisms and means that allow their protection against the phenomena of biodeterioration or biodegradation (ZHANG et al., 2018).

In order to colonize the lithic surface, microorganisms need water and mineral nutrients. Solar energy allows the absorption of CO_2 and helps to colonize the surface in the form of a biofilm (GRIFFIN et al., 1991), the stage in which favorable conditions are created for the emergence of biogeochemical circuits of other biogenic elements such as nitrogen and sulfur (FERNANDERS, 2006).

Apart from these mechanisms of colonization and biodeterioration, extracellular enzymes and the metabolism of microorganisms that develop in the microenvironment on the surface of the rock also play an important role. Among these, superoxide dismutase (SOD) plays an essential role in the metabolism of reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide and hydroxyl radicals ($O_2^{\bullet -}$, $OH^{\bullet}$), nitric oxide (NO) or hydrogen peroxide (H_2O_2) (MEHLA et al., 2017). This metalloenzyme, which can contain Cu^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+} (ROYCHOWDHURY et al., 2019) together with alkaline phosphatase can provide adaptive support as a mechanism for the development of microorganisms on lytic support. The diversity of the molecular forms of SOD demonstrates the importance of the metabolism of reactive oxygen species but also the involvement in other metabolic processes, the synthesis of the exopolysaccharide capsule and the biofilm as well as the cell morphology (NAJMULDEEN et al., 2019).

On the other hand, these enzymes can be used as indicators of the effectiveness of new methods of removing pollutants from household water that can affect aquatic life and human subjects. Thus, for the treatment of wetland surface

areas coupled with microbial electrochemical systems along with chlorophyll and total protein content, SOD activity can be used as an indicator of the efficiency of the purification system (QIU et al., 2022). Alkaline phosphatase has an important action in the regulation of various metabolic processes, being studied especially in higher animals (Chaudhuri et al., 2013), but it can be considered that, in the environmental stress conditions provided by the lytic support, it can have a particular role in the development of the microbial community that grows and multiplies in this context.

This paper is a holistic approach for SOD activity of lytic microorganisms and the types of substrate they can colonize, particularly volcanic tuff rock on. The investigated strains have been isolated from a volcanic tuff rock in the Teisani and Malul Alb areas and according to the author's knowledge this is the first study in these ecosystems based on the SOD activity approach.

MATERIALS AND METHODS

The *Culture medium* used in the experiments was represented by MH with following composition (g/L): glucose (1), proteose-peptone (5), yeast extract (10), NaCl (100), $MgCl_2 \cdot 6H_2O$ (7), $MgSO_4 \cdot 7H_2O$ (9.6), $CaCl_2 \cdot 2H_2O$ (0.36), KCl (2), $NaHCO_3$ (0.06), NaBr (0.026) in 1000 mL distilled water (VENTOSA et al., 1989; RUGINESCU et al., 2018).

Determination of catalase and oxidase activity. Catalase was assayed according to previously described method (AZHAR et al., 1995) by shuffle a 3% H_2O_2 solution with a fresh bacterial culture. The appearance of gas bubbles was considered a positive reaction. The oxidase test was performed based on phenylenediamine oxidized to indophenol and reaction is considered positive when a dark purple colour appears (SHIELDS & CATCHART, 2010).

Obtaining the enzyme preparation. The standardized inoculum (fresh 24-hour culture – 1/9 ratio) (20 ml medium with 200 µl fresh inoculum) was incubated with shaking at 30^0C for 18 hours. Afterwards, centrifugation was performed at 6000 rpm for 15 minutes, at 4^0C. Bacterial cells were washed with 0.1 M potassium phosphate buffer, pH 7.5 (2 ml) and resuspended in the same amount of solution. The suspension was sonicated (on ice) and then centrifuged at 12000 rpm for 20 minutes at 4^0C. The supernatant was saved for determination of total protein and superoxide dismutase.

The total protein content has been determined by Lowry method.

Superoxide dismutase was assayed by the Winterbourn method (1975) which is based on the ability of these enzymes to inhibit the reduction of the Nitro Blue Tetrazolium salt by the superoxide radicals formed as a result of riboflavin reduction. The reaction mixture contains: 2.55 mL of phosphate buffer 36 mM and pH 8; 0.20 mL EDTA 0.1M, pH 7; 0.10 mL enzyme preparation; 0.10 mL nitro blue tetrazolium 1.5mM and 0.05 mL riboflavin 0.12 mM. After vigorous shaking and illumination 5 minutes the extinction at 560 nm against distilled water is recorded. Similarly, a blank mixture is prepared without enzyme preparation. The blank volume was adjusted with phosphate buffer. The unit of superoxide dismutase activity is calculated according to the formula $S = [(100\text{-}Ep)x2]/(Em x A)$ where Ep - sample extinction; Em - the extinction of the blank and A – the amount of protein in the sample, respectively in 0.1 ml of liquid to be analyzed.

To determine the *alkaline phosphatase activity*, one gram of rock was mixed with 4 mL TrisHCl 0.05 M, pH 11 and 1 mL p-nitrophenylphosphate 0.115 M was added. The mixture was kept for one hour at 37^0C in a water bath. Later, 1 mL of 0.5 M $CaCl_2$ and 1 mL of 0.5 M NaOH were added. The resulting mixture was filtered and the concentration of p-nitrophenyl was determined spectrophotometrically at 400 nm. The enzyme concentration was expressed as µmols/h/mL.

RESULTS

The investigated samples were represented by several rock fragments or water flow which levigates the areas from the Teișani, Prahova county and Malul Alb, Buzău county (Fig. 1). The sampling site of Teișani is located nearto the Slanic halite deposit and Malul Alb in the proximity of the Meledic saline plateau. Previous data (BĂTRÎNESCU-MOTEAU et al., 2022) revealed a relatively high number of colonies forming units in investigated samples from which randomly were selected a number of isolates for further purification. After successive passage on slant medium or during to some experiments several of them (around 5%) lost capacity to grow and 64 isolates were kept for investigation.

These bacterial strains isolated from a volcanic tuff rock were tested for their enzymatic activities of superoxide dismutase as support for the metabolic reactions to response for the adaptation of hard conditions for life either to the surface of inside of the rock. Out of these strains, 47 strains have a positive answer for catalase activity and 16 remain with a negative one. In the case of the oxidization test, similar numbers were recorded in both answers but the strains were different (Fig. 2; Table 1).

The protein content resulted from enzyme preparation varying from 0.35 (strain P.A.-3 isolated from Malul Alb rock sample) until to 7.18 mg/mL (strain APA-4 isolated from levigate rain water from the Teișani investigated area). These concentrations support a highly superoxide dismutase (SOD) enzymatic activity, also considering the number of isolated microbial strains. The recorded data revealed a wide spectrum of SOD values activity ranging from 136,7637 U/mg of protein, sample T3-5 isolated from Teisani rock sample until to 6628,25 U/mg, sample P.A.-3 isolated from the Malul Alb rock sample (Figs. 3; 4). Most of the values within this range are close to the lower limit.

Figure 1. The overview of the sampling sites of volcanic tuff rock. Top, Teișani and bottom Malul Alb areas (original).

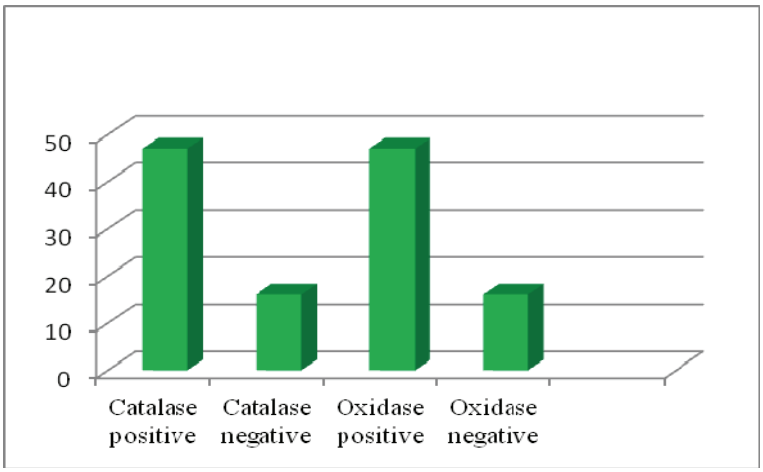

Figure 2. The number of strains with catalase/oxidase positive or negative activities.
The number of investigated strains is represented on the Y axis.

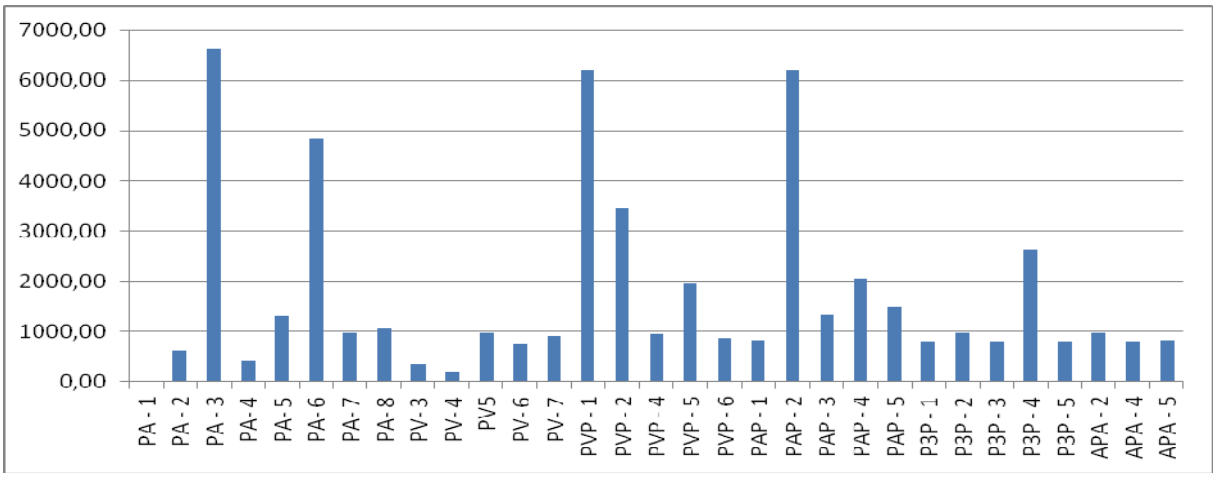

Figure 3. The range of superoxide dismutase (SOD) values for investigated strains. The SOD values (U/mg) are represented on the y-axis and the investigated strains on the x-axis.

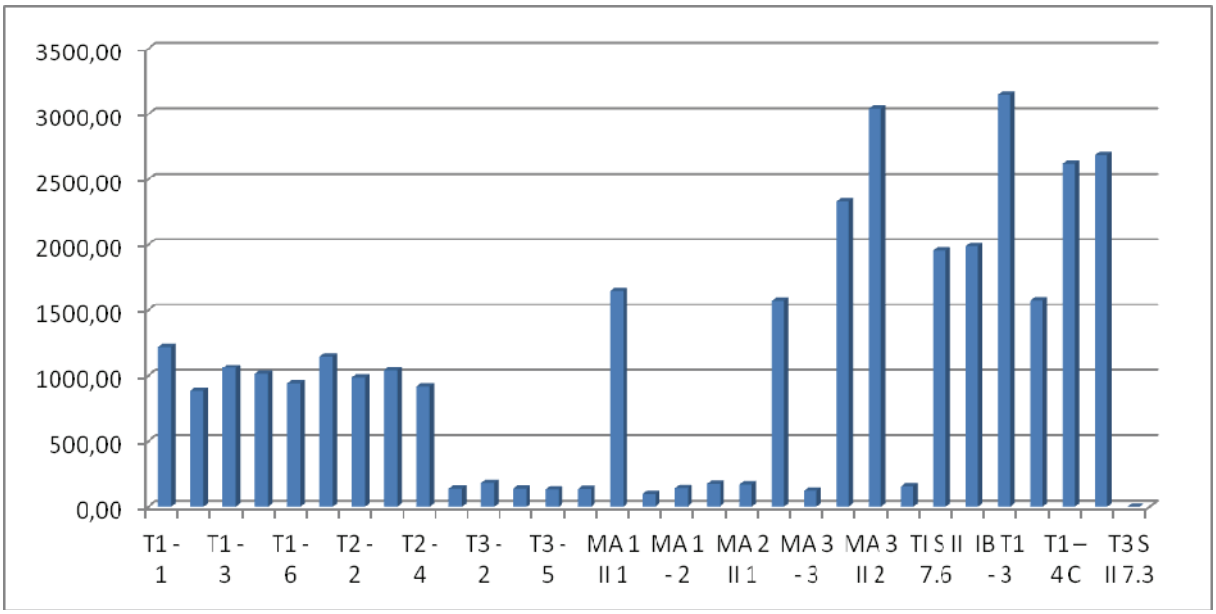

Figure 4. The range of SOD values for investigated strains. The SOD values (U/mg) are represented on the y-axis and the investigated strains on the x-axis.

When compared with SOD values considered normal for humans, for example, which are expected to range from 1200 to 2000 U/mg (PIRGARI, 2016; ZHANG et al., 2002), 14 from our isolates (22,5%) showed higher values than scale, 9 isolates have normal (14.5%) and 39 lower (63%) values. On the other hand, if compared with plant scale with SOD values between 220 and 300 U/mg (LU & YEAP FOO, 2001), 50 isolates (80.6%) have high, 1 isolate (1.6%) have normal and 11 isolates (17.8%) have lower activities. Conversely, when compared with animals scale with SOD values between 1992 – 5970 U/mg (TODOROVA et al., 2005), the percentage of isolates with lower values is predominant, 48 isolates (77.5%). The normal values corresponding for 11 isolates (17,7%) and 3 isolates (4.8%) have higher values (Fig. 5). In general, when the strain isolation medium was supplemented with peptone, an increase in SOD activity was observed in all cases due to the acceleration of the oxidative metabolism of the isolated strains. A single exception was recorded for the P.A.3 strain isolated from the volcanic tuff rock from Malul Alb. The obtained results are similar both for the strains isolated from the volcanic tuff rock from Malul Alb and from the one from Teisani (Figs. 6; 7). The investigated strains were isolated from 13 variants of rock samples belonging to several sampling points. Thus, in the case of the sample from Malul Alb, there were 7 variants of isolation media and for the volcanic tuff rock sample from Teișani, 6 variants. The average values of SOD activity in these cases vary from 159.29 U/mg, sample MA 4 to 2686.13 U/mg in the case of sample PVP (Fig. 8).

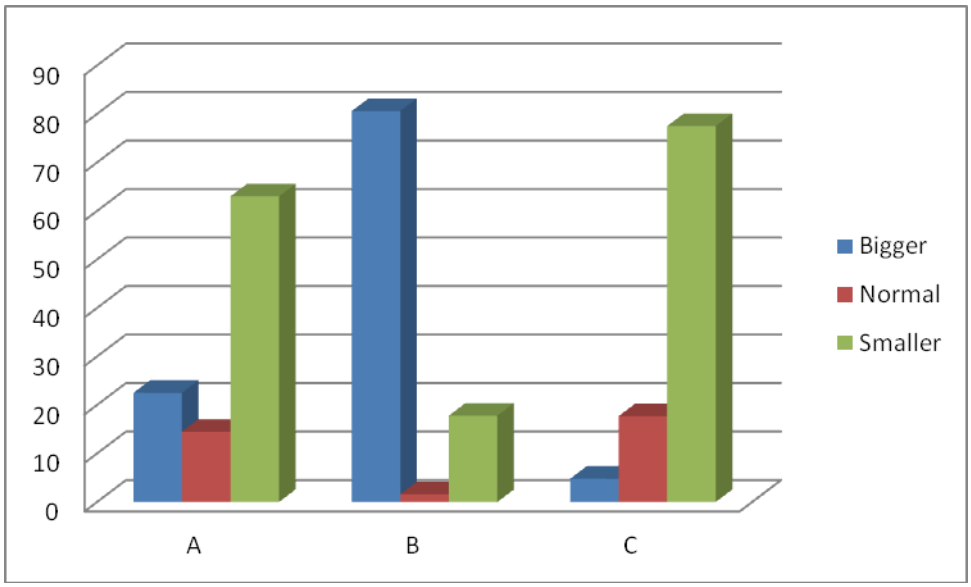

Figure 5. The percentage of strains with SOD values reported to human subject (A), plant (B) and animal (C) values. The percentage values are represented on the y-axis.

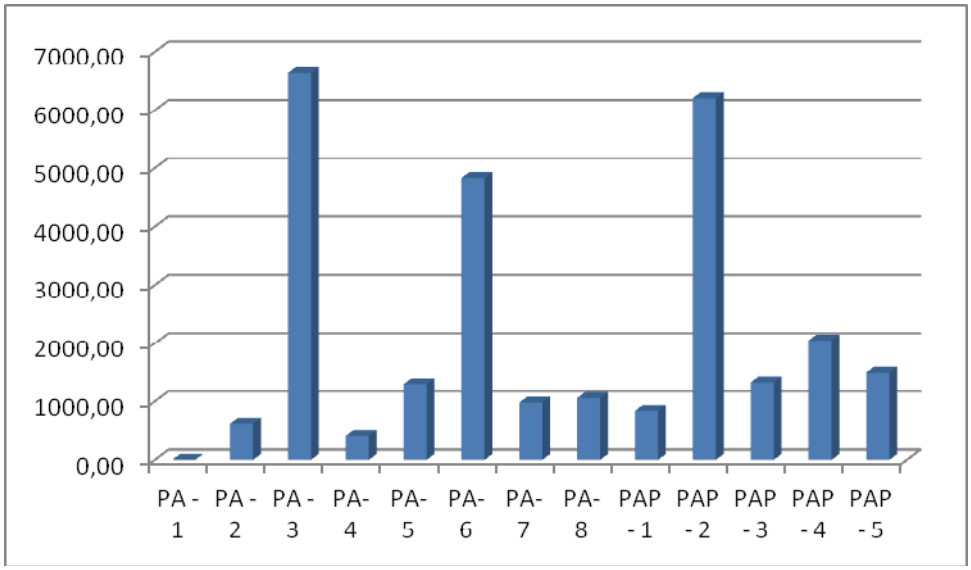

Figure 6. The influence of peptone adding on the SOD values (Malul Alb strains). The SOD values (U/mg) are represented on the y-axis and the investigated strains on the x-axis.

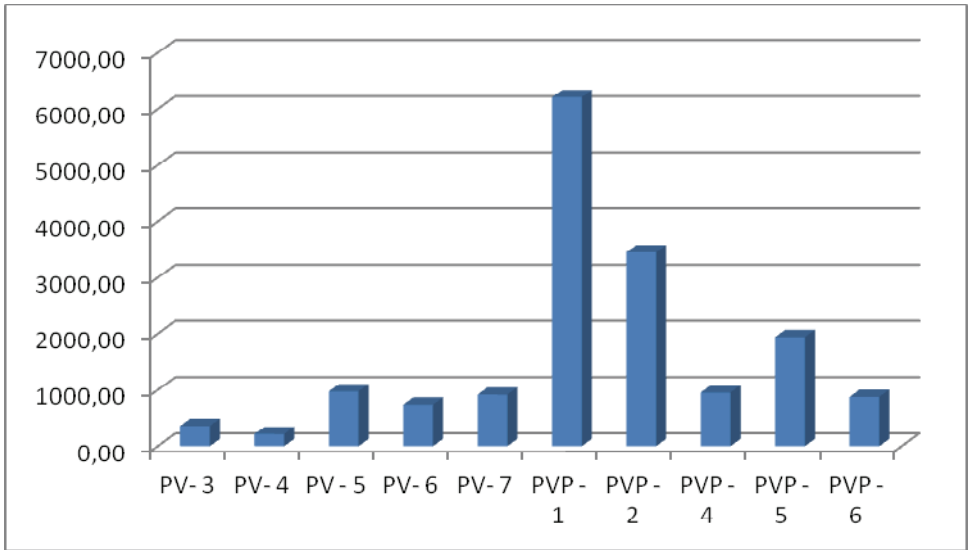

Figure 7. The influence of peptone adding on the SOD values (Teisani strains). The SOD values (U/mg) are represented on the y-axis and the investigated strains on the x-axis.

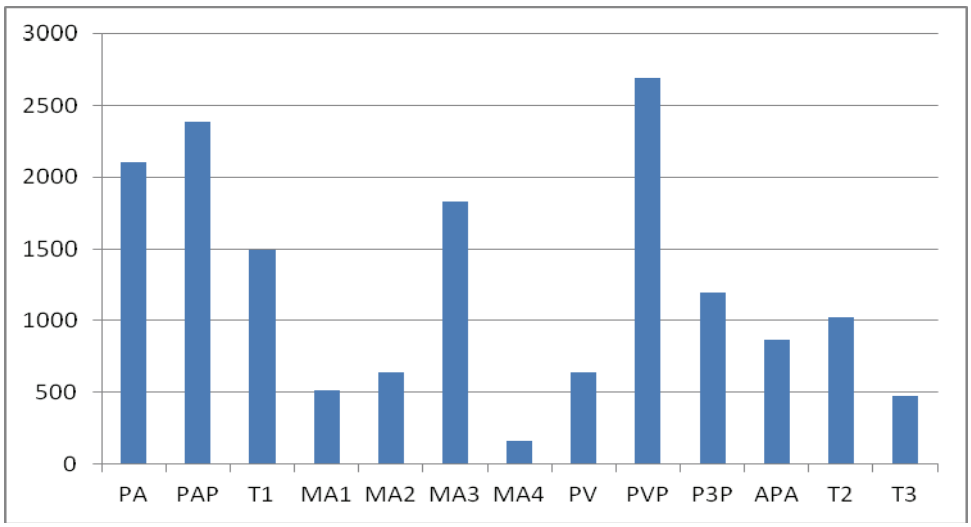

Figure 8. Comparative media values of SOD of the rock sample from Malul Alb (PA, PAP, T1, MA1-MA4) and Teișani. The SOD values (U/mg) are represented on the y-axis and the investigated strains on the x-axis.

The obtained data show a higher activity of SOD in the case of the samples from Malul Alb. The two isolation sites differ in terms of anthropogenic impact, the Malul Alb area being an isolated one, difficult to access compared to the exposed Teisani area in terms of geography and tourist attraction. In terms of chemical composition, they are similar (BĂTRÎNESCU-MOTEAU et al., 2022) and as regards the metallic ion in the SOD composition found in the two rock categories tested, this is manganese, with an average concentration of 0.3 mass% in the case of the rock sample from Teișani and 0.2 mass% for Malul Alb. In the analyzed sample taken from the Teișani area, Cu^{2+}, 0.08 mass% and Zn^{2+}, 0.04 mass% were also identified (BĂTRÎNESCU-MOTEAU et al., 2022; 2023) as cofactors of SOD metalloenzymes.

On the other hand, SOD and alkaline phosphatase (AP) activities in the investigated rock samples significantly fluctuated (Fig. 9; Table 1), which indicated an adaptive physiological mechanism when challenges appear (Liang et al., 2020) at the surface of rock environments, where microbial communities predominantly are developed as biofilms or like a subaerial biofilm at the interface of rock with atmosphere (GORBUSHINA, 2007). The recorded data showed that relatively high values for SOD activities are associated with smaller values for AP activities. A similar behaviour was also reported as protection mechanism by reducing the oxidative stress generated by reactive oxygen species and restoroffing the nitrogen compounds with a beneficial role for the organism (YIN et al., 2012).

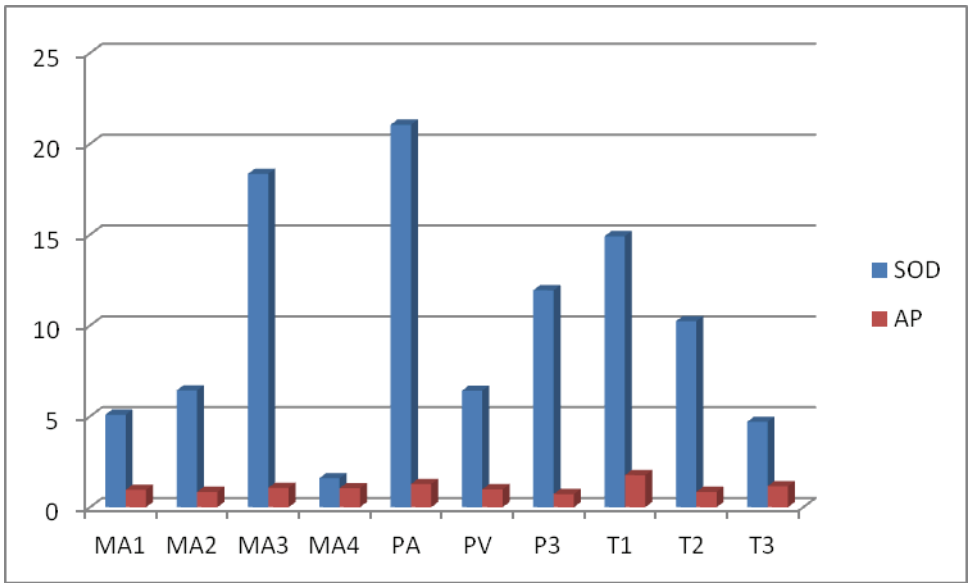

Figure 9. SOD and alkaline phosphatase (AP) activities of the investigated rock samples. For SOD values, a multiplication with 10^2 is necessary. On the Y axis are represented the values of the two enzyme activities expressed in U/mg (SOD) and μmols/h/mL (AP).

Table 1. The bacterial strains isolated from the investigated areas of Malul Alb and Teișani. The codes are assigned as follow: P.A., MA and T1 for Malul Alb rock sample; P.V., P3 and T for Teișani rock sample. APA representing levigate rain water in Teișani area. Codes with P (i.e. PAP) are supplemented with peptone for microbial strains isolation. The associated number represents a different sampling point in the same area.

Isolated strain identification code	Volcanic tuff rock source	Protein concentration (mg/mL)	Superoxide dismutase activity (units/mg of protein)	Catalase reaction results	Oxidase reaction results
P.A. - 1	Malul Alb	2.32	999.97	+	+
P.A. - 2	Malul Alb	3.72	623.58	+	+
P.A. - 3	Malul Alb	0.35	6628.25	+	+
P.A. - 4	Malul Alb	5.51	421.02	-	+
P.A. - 5	Malul Alb	1.78	1303.27	+	-
P.A. - 6	Malul Alb	0.48	4831.19	+	+
P.A. - 7	Malul Alb	2.37	978.67	+	+
P.A. - 8	Malul Alb	2.20	1054.4	+	+
P.V. - 3	Teișani 1	6.48	357.96	+	+
P.V. - 4	Teișani 1	5.41	214.39	+	+
P.V. - 5	Teișani 1	5.75	974.2911	+	+
P.V. - 6	Teișani 1	3.13	741.18	+	+
P.V. - 7	Teișani 1	2.53	916.86	+	+
PVP - 1	Teișani 1	1.79	6207.225	+	+
PVP - 2	Teișani 1	3.22	3450.545	-	+
PVP - 4	Teișani 1	5.87	954.3976	+	+
PVP - 5	Teișani 1	5.69	1942.218	+	+
PVP - 6	Teișani 1	5.46	876.3035	+	+
PAP - 1	Malul Alb	5.70	839.3377	+	+
PAP - 2	Malul Alb	1.79	6207.126	+	+

PAP - 3	Malul Alb	4.20	1333.804	+	-
PAP - 4	Malul Alb	5.42	2049.988	+	+
PAP - 5	Malul Alb	3.19	1499.807	+	-
P3P - 1	Teișani 2	7.10	788.9936	+	+
P3P - 2	Teișani 2	5.79	967.5641	+	+
P3P - 3	Teișani 2	6.10	784.2937	+	+
P3P - 4	Teișani 2	4.21	2639.129	+	+
P3P - 5	Teișani 2	7.09	790.1467	+	+
APA - 2	Teișani 3	4.86	984.4399	+	+
APA - 4	Teișani 3	7.18	780.2119	+	+
APA - 5	Teișani 3	5.76	830.617	-	+
T1 - 1	Malul Alb	4.60	1217.882	-	+
T1 - 2	Malul Alb	6.31	887.8611	-	+
T1 - 3	Malul Alb	5.28	1061.03	-	+
T1 - 5	Malul Alb	5.53	1013.014	-	+
T1 - 6	Malul Alb	5.94	943.1542	+	-
T2 - 1	Teișani 1	4.88	1147.888	-	+
T2 - 2	Teișani 1	5.68	986.1374	-	+
T2 - 3	Teișani 1	5.39	1039.327	-	+
T2 - 4	Teișani 1	6.09	919.8466	-	+
T3 - 1	Teișani 2	3.22	142.6891	-	+
T3 - 2	Teișani 2	2.49	183.923	+	+
T3 - 3	Teișani 2	3.21	143.121	-	+
T3 - 4	Teișani 2	-	-	+	+
T3 - 5	Teișani 2	3.36	136.7636	-	+
MA 1 - 1	Malul Alb	3.30	139.3109	+	+
MA 1 II 1	Malul Alb	6.74	1648.44	+	-
MA 1 II 2	Malul Alb	4.44	103.3701	+	+
MA 1 - 2	Malul Alb	3.15	145.9589	+	+
MA 2 - 3	Malul Alb	2.57	178.6565	-	-
MA 2 II 1	Malul Alb	2.64	173.5089	+	-
MA 2 II 2	Malul Alb	7.05	1575.893	-	-
MA 3 - 3	Malul Alb	3.61	127.2835	+	-
MA 3 II 1	Malul Alb	4.76	2333.842	+	-
MA 3 II 2	Malul Alb	3.65	3043.053	+	-
MA 4 II 1	Malul Alb	2.87	159.2985	+	-
TI S II 7.6	Teișani 1	5.68	1956.127	+	-
T1 S II 7.12	Teișani 1	5.59	1987.279	+	+
IB T1 - 3	Teișani 1	3.53	3145.175	+	-
T1 – 1C	Teișani 1	7.04	1578.108	+	+
T1 – 4C	Teișani 1	4.24	2620.189	-	-
T3 S II 7.2	Teișani 2	4.14	2683.188	+	-
T3 S II 7.3	Teișani 2	3.93	2826.22	+	-

CONCLUSIONS

The 64 investigated strains isolated from volcanic tuff rock showed SOD activity in a wide range between 136.7637 U/mg and 6628.25 U/mg. Compared to the values considered normal for SOD activity in human subjects, 22.5% of the strains had a higher value, 14.5% normal and 63% lower. Compared to the values related to plants, 80.6% of the strains had a higher value, 1.6% normal and 17.8% lower and in the case of the values for animals 48% had lower values, 17.7% normal and 4.8% higher. Higher values of SOD activity are associated with lower values of alkaline phosphatase activity and their fluctuation shows an adaptive mechanism to the microclimate on the surface of the volcanic tuff rock.

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