NEW DATA CONCERNING THE PRESENCE OF MICROORGANISM IN A SALT SPRING – A CASE STUDY SALT SPRING "LA MURĂTOREA", CĂNEŞTI VILLAGE, BUZĂU COUNTY, ROMÂNIA

MĂDĂLIN ENACHE[1], CAMELIA MUȘAT[1], ADINA TUZLARU[1],
COSTIN BĂTRÎNESCU-MOTEAU[1], MIRELA ENACHE[2], GABRIEL MARIA[1]

Abstract: The current study pays attention to microbial composition for a salted spring located in Buzău County in România. The results revealed a poor composition in microorganisms only 22 cultivable isolated being detected. The 11 isolates were selected from which six are catalase and two oxidase positive at these tests. All are Gram positive. One isolate is an actinomycete and the rest are bacteria. Two isolates are white colour, another two are red colour and one is orange. The pigmentation can be considered as factor with biological activity. The investigated isolates have a coccus shape either individual or in aggregate forms. The spectral analysis of the investigated samples reveals that the UV absorption spectra are characteristic for carotenoid precursors like phytoene and early polyenes.

Keywords: salt spring, halobacteria, salted environments, saline pigments.

INTRODUCTION

The microorganisms which inhabit saline and hypersaline environments either in liquid forms like salted lakes, salted springs etc. or in solid forms like salt deposits, evaporates and salt mining areas are called halophiles. They are mainly represented by bacteria or archaea but some fungi were also discovered in these types of extreme ecosystems (Oren, 2002). On the other hand, another kind of salted environments are represented by salted springs which are also populated by halophilic microorganisms (Diaconu, 2016). As a general rule the salted springs discharge in groundwater (Gou *et al.*, 2019) and are responsible for its mineralization, but if they appear at the surface it will discharge in the water from proximity and contribute mainly at the salinity and the composition of microorganisms in this water.

[1] Institute of Biology Bucharest of the Romanian Academy, 296 Spl. Independentei, Sect. 6, 060031, Bucharest, Romania, e-mail: madalin_enache@yahoo.com
[2] Institute of Physical – Chemistry „Ilie Murgulescu", 202 Spl. Independentei, Sect. 6, 060021, Bucharest, Romania.

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Salted springs beginning terrestrial surface water are supposed to anthropic impact and in this way became polluted water. On the other hand, the putative pollution can have the origin from the spring circuit or by the aerial fall of particles. Another study suggested that the putative pollution of salt springs is apparently not correlated with anthropogenic pollution (Playa *et al.*, 2024).

Such salted springs that appear at the soil surface are widely distributed on Romanian territory and they're used for various purposes, such as recreational areas or alternative therapies. (Alexianu *et al.*, 2008). Such an area well known for its salty springs is Buzau County.

Buzău County is located in the southern-east part of Romania, approximately 100 km from the capital Bucharest. The Cănești village is located in the central-north area of Buzău county, being characterized by the presence of several salt resources such as the Meledic plateau, the Lopătari area characterized by the presence of some salty springs, the salty river "Sărătel" with which the spring "La Murătorea" has a direct connection. More than 22 salt springs were evaluated in a study of Doina Ciobanu, a passionate researcher for the salted area in this geographical area (Doina Ciobanu, 2010). The spring is used by the population of the village and from the vicinity of the area for the preparation of some preserved salty foods throughout the year, and during the winter they give this water to animals such as cattle and other ruminant animals. Also, during the summer, the local population uses the water from the Sărățel river for bathing. Even though numerous studies have been conducted on this salty spring in recent years, little is known about the microbiota and their potential for the use of biotechnologies and for the exploitation of economic values of microorganisms present in such waters considered extreme ecosystems that are little investigated from a microbiological perspective.

Considering that such types of salted waters are used in human alimentation (Alexianu *et. al*, 2008; Trajković Pavlović *et al.*, 2015), it is important to have knowledge about the microbial composition.

The present study pays attention to the microbial community in this salted spring and its putative potential for various purposes like pigment synthesis which is secreted extracellularly, and spectral characterization of the pigments.

MATERIALS AND METHODS

The samples were taken in the summer of the year 2024 from the investigated spring and kept at refrigerator until transportation to the laboratory. The samples were represented by water from the spring, mixture with soil from the point in which water touch the soil, mixture of crystallized salt with soil and air from the proximity. In order to take air sample a Petri dish has been open for 10 minutes and then covered with sterile aluminium foil and with lid. Into laboratory the lid has been removed and approximatively five mL of air has been extracted and mixed in five mL of culture media.

The selected isolated were grown on the culture media supplemented with NaCl until to 4M concentration and the growth has been evaluated by registering optical density at 660nm.

The chemical composition of the sample was carried out by XRF analysis according to previous descriptions (Bătrînescu-Moteau *et. al.*, 2022), using the Rigaku ZSX100e Supermini XRF system (Rigaku, Japan), following the manufacturer's protocol (Lewis *et. al.*, 2012).

The presence of catalase was highlighted by mixing the bacterial culture with hydrogen peroxide and if gas bubbles appear the reaction is judged as positive on. To determine the presence of oxidase the "oxidase reagent" – N,N-Dimethyl-p-phenylenediamine hydrochloride – was used. Once the reagent is oxidized the colour of the reagent, initially colourless, evolves to shades of dark blue or purple (Cathcart L. and Shilds, P., 2010).

The Gram character was evaluated using the potassium hydroxide test (Buck, 1982).

The investigation of the morphology of the microbial strains was performed by optical microscopy, using a Zeiss Axio Imager.M2m microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), equipped with a digital camera for capturing and recording microscopic images.

Smears from pure cultures of microbial isolates were prepared for analysis, according to the methodology described by M. Licker (2019).

The analysis of the isolates was performed by scanning electron microscopy (SEM), using a Jeol JSM-6610LV microscope (JEOL Ltd., Japan). A volume of 10 µl of suspension was deposited on a microscope slide, then the surface of the sample was coated with a thin layer of gold, by sputtering. The slides were dried in vacuum, and subsequently metallized by depositing a conductive layer using the JFC-1300 gold coating device (coater).

UV–VIS investigations were carried out on a JASCO V-630 spectrophotometer equipped with a Peltier-controlled ETCR-762 accessory (JASCO Corporation, Tokyo, Japan) in 200 – 800 nm spectral region. The experiments utilized a matched pair of quartz cuvettes with an optical path length of 1.0 cm. For the investigations, the pigments were dissolved in ethanol reagent grade.

RESULTS AND DISCUSSION

The chemical analysis of the investigated samples revealed a chloride content of 202,35 g/L, close to the maximum solubility of sodium chloride in water and follow chemicals elements: Al_2O_3 – 10,06%; SiO_2 – 73,99%; K_2O – 4,73%; CaO – 5,17%; Fe_2O_3 – 3,65%; TiO_2 – 1,03%, and a trace of lanthanides and actinides. The high percent of silicon shows that the depth of the spring is great.

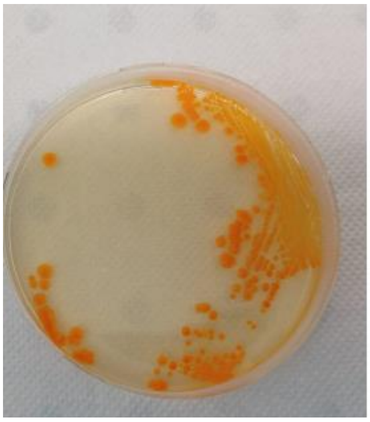
Table 1

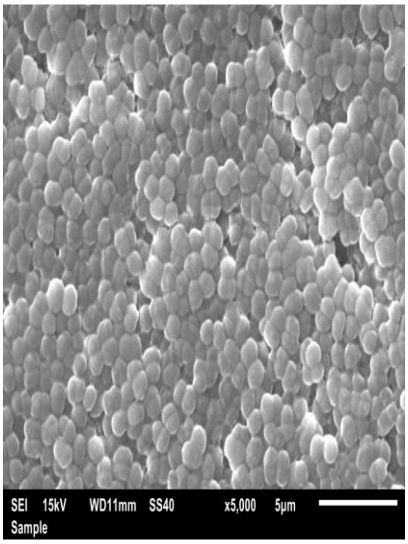
The summary characterization of investigated selected isolates. A = mixture with soil from the point in which water touch the soil, B = air from the proximity of the spring, C = mixture of crystallized salt with soil

Isolate	Source of isolate	Gram test (KOH 3%)	Catalase activity	Oxidase activity
G3 10^5 12 white	C	+	-	+
G3 10^5 12 red	C	+	-	-
CMC 2	B	+	+	-
G3 10^2 11 white	C	+	-	-
G1 10^2 14	A	+	+	-
MH 1 10^1 1	A	+	+	-
2	B	+	-	+
CMC 1	A	+	+	-
G3 10^2 10 Act	C	+	+	-
G3 10^2 11 Red	C	+	-	-
CMC 3	C	+	+	-

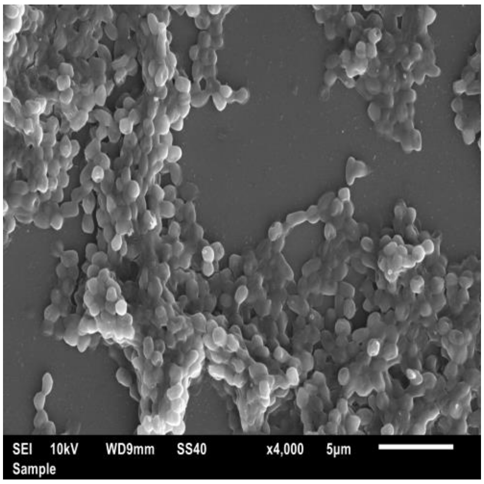
From the investigated strains were selected 11 which presented various degree of pigmentation (Figs. 1 and 2) from which were performed supplementary investigations. All the isolates are Gram positive (Table 1), six are catalase positive and two are oxidase positive. The data recorded on Table 1 show that the isolates which are oxidase positive are catalase negative. The isolate from the air sample from the proximity of spring are only oxidase positive. The data obtained either by optical microscopy or scanning electron microscopy (SEM) investigations showed that investigated isolates have a coccus shape either individual or in aggregate forms (Figures 1 and 2).


(A)


(B)



(C)



(D)

Figure 1. The data revealing shape of some isolates (B, C, D), pigmentation (B) and water sample taken from the spring (A).

The data from the literature (Selvameenal *et al.*, 2009; Quintero-Pacheco *et al.*, 2024) revealed that pigment synthetized by several microorganisms including also actinomycetes have antimicrobial activity towards pathogens bacteria. In this frame will be expected that pigments obtained from the isolates from “La Murătoarea” salt spring to have some biological activity or other roles in the environment in which they are released.

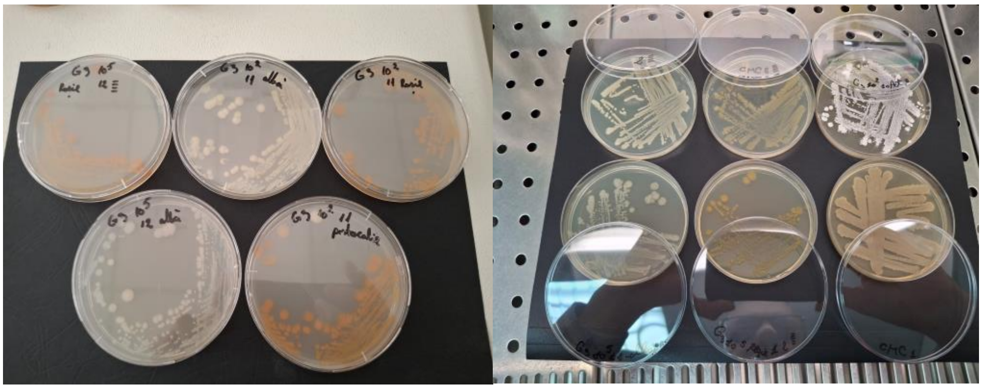


Figure 2. The pigmentation varying from white to orange and red of the investigated isolates.

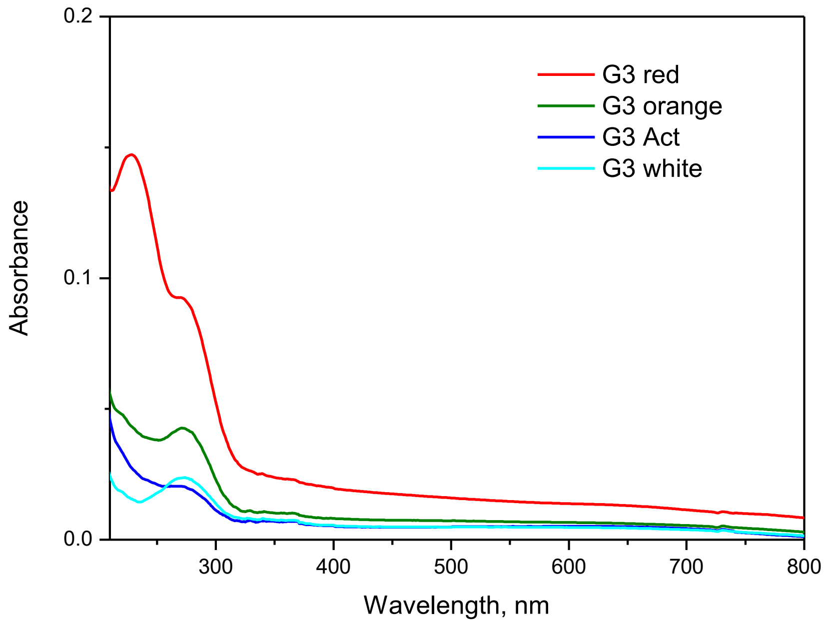
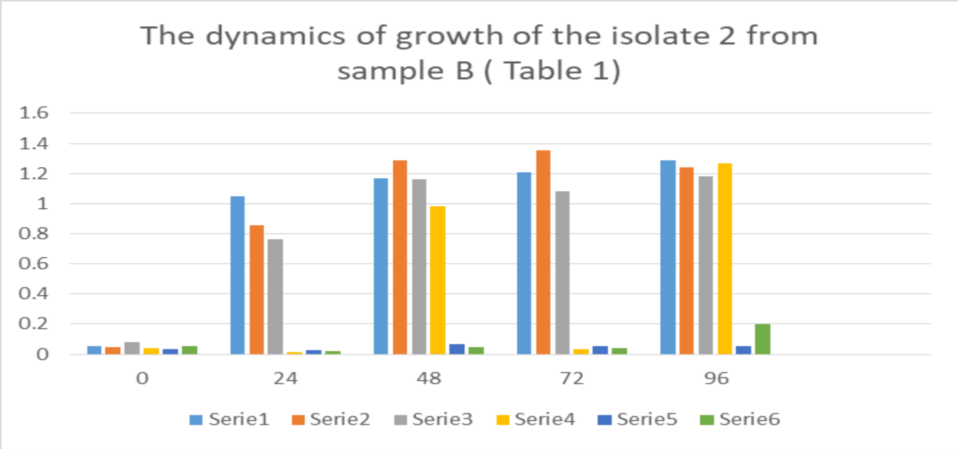


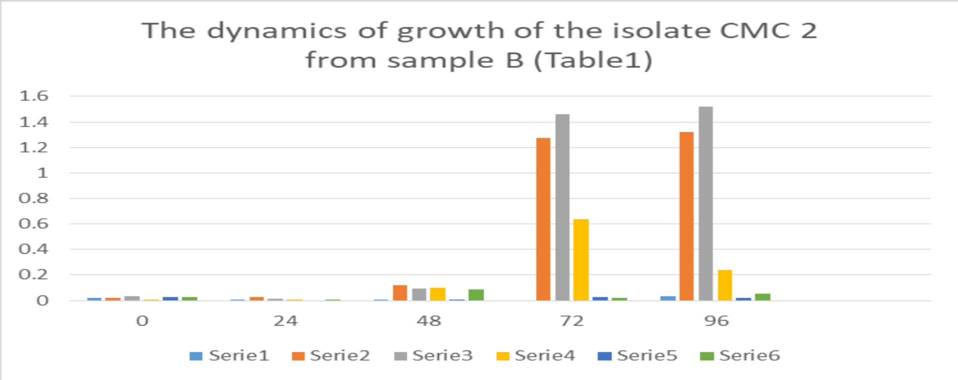
Figure 3. The UV-VIS absorption spectra of the investigated ethanol diluted samples in the range of 210 – 800 nm.

Figure 3 displays the absorption spectra of the investigated samples diluted 100 times in ethanol. It can be observed that all samples present maximum absorption peaks in UV region despite their slight red or orange coloration. Thus, the sample G3 red presents an absorption peak at 227 nm and a shoulder at 269 nm. The samples G3 orange, G3 white and G3 Act present a maximum absorption peak at about 271 nm.

The absence of any absorption bands in the 400 – 500 nm wavelength region (which are characteristic for the most carotenoids) [*HarvestPlus Handbook for Carotenoid Analysis, D.B. Rodriguez-Amaya, M. Kimura; HarvestPlus Technical Monograph Series 2; IFPRI: Washington, DC, USA, and CIAT: Cali, Colombia, 2004*] indicate the absence of carotenoids pigments in the investigated samples.

Absorption at ~ 270 nm is often due to $\pi \rightarrow \pi^*$ transitions of aromatic systems and some conjugated double bonds. In our samples, the absorption spectra are typical for phytoene (three conjugated double bonds), which are carotenoid precursors in the carotenoid biosynthetic pathway. Also, the absorption peak at about 227 nm for G3 red sample is characteristic for carotenoid precursors such as early polyenes (simple conjugated diene with two conjugated double bonds).





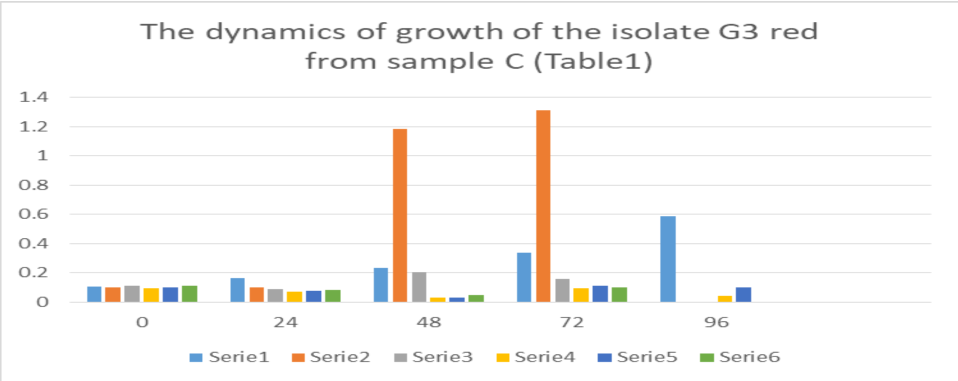


Figure 4. The dynamics growth of some selected isolates in the presence of NaCl in growth medium al NaCl concentrations varying from 0 until to 4 M. Series 1 – 0M NaCl, Series 2 – 0.5M NaCl, Series 3 – 1M NaCl, Series 4 – 2M NaCl, Series 5 – 3M NaCl, Series 6 – 4M NaCl. Abscissa axis representing the time in hours of cultivation and ordinate axis representing optical density at 660 nm.

The data in figure 4 represent the dynamics of the development of selected isolates, namely G3 red and 2 (Table 1) depending on the NaCl concentration in the growth medium. An influence of salinity can be observed in the case of the two selected isolates.

In the case of isolate 2, originating from the air sample from the proximity of the studied salty spring, higher NaCl concentrations prevent its development. This may suggest that in the atmosphere the aerosol content is not very high even if the spring salinity is high. In the presence of carboxymethyl cellulose (CMC) in the cultivation medium the aspect of the growth dynamics is similar but is slowed down in the first 48 hours of growth. This behavior could be due to difficulties in adapting the isolate to the presence of several carbon sources, considering that it is isolated from the atmosphere.

The red G3 isolate originating from the sample marked C in Table 1 (contact between spring water and soil) shows a similar dynamics to isolate 2, noting a more intense development in the interval 48–72 hours in the presence of 1M NaCl. After this interval, the isolate practically stops developing. Most likely, upon contact with soil, the NaCl content of the spring water decreases.

CONCLUSIONS

The data recorded in this study showed that the investigated sample have a low microbial load. This can be argued by high chloride concentration of the water and by the depth of the spring. From the selected isolates some showed various degree of pigmentation from white or red colour. The pigmentation can be considered as factor with biological activity (Selvameenal *et al.*, 2009). The isolates belonging to bacteria, fungi and actinomycetes. The investigated isolates have a coccus shape either individual or in aggregate forms.

The spectral analysis of the investigated samples reveals that the UV absorption spectra are characteristic for carotenoid precursors like phytoene and early polyenes.

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