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Evaluation of Halobacterial Extracellular Hydrolytic Activities in Several Natural Saline and Hypersaline Lakes from Romania

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Authors' contributions

This work was carried out in collaboration between all authors. Author ME designed the study, wrote the protocols and wrote the first draft of the manuscript. Authors RC and IP managed and performed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This work presents the evaluation of extracellular hydrolytic activities involved in the degrading of organic compounds from several natural salt lakes located in the southern part of Romania. The paper approaches the extracellular enzymatic decomposition activity in accordance with several physico-chemical parameters and microbial abundance. The chloride content was 13 g/l in Balta Albă salt lake, around 15 – 17 g/l in Amara salt lake, 80 g/l in Movila Miresei hypersaline lake and close to saturation, 252 g/l respectively in the case of Ocnele Mari hypersaline sample. The chemical composition revealed that salinity is due to compounds of sulphur, chloride and potassium in lakes Balta Alba and Amara. In Movila Miresei, potassium is replaced by sodium and in Ocnele Mari the salinity is due mainly to chloride and sodium. The enzyme α –glucosidase showed high values in the hypersaline lakes Movila Miresei and Ocnele Mari and a similar profile was recorded also for β-glucosidase. The activity of the enzyme alkaline phosphatase is directly correlated with the chloride content. The enzyme aminopeptidase showed high values if compared with previously mentioned enzymes.

Keywords: Salt lake; extracellular hydrolases; halophilic microorganisms; halophilic enzyme.

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1. INTRODUCTION

Salted lakes are generally regarded as harsh environments for the growth of microorganisms [1,2]. Considering this specific overview, few studies were oriented in their well-structured research even if in the last years abundant information appears oriented mainly to microbial biodiversity of these environments [3]. This biological diversity covers microorganisms belonging to both *Archaea* and *Bacteria* domains, but also some new eukaryotic species were described as inhabitants of salted areas (mainly lakes) in the last period. More than 150 archaeal species distributed in 40 valid genera were described as populating saline and hypersaline environments [3,4]. When using the term hypersaline, microbiologists generally consider the known salt lakes like the Dead Sea, Great Salt Lake or some salt mines [5,6]. In these environments the content of sodium chloride is higher than 100 g/l, but there are also some salt environments where the sodium chloride content varies from modest content of 15 g/l till saturation. In our study we considered environments with chloride content between 3 g/l and 35 g/l are saline environments, over 36 g/l were regarded as hypersaline environments and bellow 3 g/l should be considered fresh waters [1,7].

The salt lakes are widely distributed in Romania where more than 100 are mentioned in literature [7–13] as natural or man-made. In the last years several species of archaeal and bacterial strains were described, most of them being capable of degrading macromolecules with compositions based on sugars or proteins, due to the presence of some extracellular enzymes [14]. Specific richness in biodiversity could be found in such kind of saline habitats which represent typical examples of extreme environments. The spectrum of living forms from these biotopes is determined by several physico-chemical parameters like chloride content, salinity and ionic content, temperature and oxygen solubility, pH value [15].

In spite of the harsh physico-chemical conditions offered by saline and hypersaline environments, several living organisms have been isolated and further investigated, among which we can mention: the brine shrimp *Artemia salina*, the brine fly *Ephydra*, photosynthetic flagellates belonging to genera *Dunaliella*, *Asteromonas*, *Synechococcus* and lot of prokaryotes either bacteria or Archaea. Such kinds of organisms are capable to grow in media with high (>0.2M) sodium chloride content being regarded as halophilic or in media without sodium chloride, but being capable to rapidly adapt to the presence of high sodium chloride concentrations are regarded as halotolerant [16]. Kushner [17] defined the term halotolerant for organisms that are able to grow in media without NaCl, but are able to grow at high salt concentrations, while the term halophilic is used for those that require the addition of NaCl in media for their growth. Oren [18] defined halophiles as “microorganisms that are able to grow well above 100 g/l salt”.

Halophilic microorganisms have the potential to be utilized for obtaining products such as β-carotenes, enzymes (amylase, lypase), polymers (poli-β-hydroxyalcanoate) and compatible solutes (ectoine). Moderately halophilic and halotolerant bacteria are regarded as effective in the treatment of waste waters from various industries [19]. The ability of halophilic microorganisms to degrade pollutants of organic and inorganic origin has been reviewed and described by Oren [18,20], Ventosa & Nieto [21] and Oncescu et al. [22]. The degradation and decomposition of organic compounds in saline ecosystems should be regarded as key processes for the good functioning of the ecosystem which are mainly due to the extracellular enzymes produced by halophilic present in these environments. This paper deals with the evaluation of extracellular hydrolytic activities involved in degrading organic compounds from several natural salt lakes located in the southern part of Romania and having various origins like the evaporation of ancient sea or the natural breakdown of some river courses [11]. The paper approaches the study of extracellular enzymatic decomposition activity in natural salt lakes in accordance with several physico-chemical parameters and microbial abundance. The salted environments complexity was described from this point of view by Javor [1] in anthropic salted environment, namely saltern Exportadora de Sal [1].

2. MATERIALS AND METHODS

2.1 Samples Sites

The water samples have been taken from several Romanian salt lakes like Amara, located in Ialomiţa county, approximately 120 km south-east of Bucharest, Balta Albă (White Bath) located at the border between counties Buzău and Brăila, approximately 150 km south-east of Bucharest, Movila Miresei (Bride Knoll) located in Brăila county, approximately 200 km south-east of Bucharest and Ocnele Mari area (High Salt Mines) in the areas with the same name located in the proximity of the city Rîmnicu Vâlcea, approximately 180 km south-west of Bucharest. The salt lakes Amara and Balta Albă are included in the European ecological network for nature conservation Natura2000, being part in site ROSPA 0004 and 0065 and ROSCI005. The physical aspects of the lakes were recorded in photo images using a digital camera Canon PowerShot model Pro 1.

2.2 Physico-chemical Analysis of the Water Samples

The investigations have been performed as previously described in order to estimate the pH, density and chloride content [23]. The concentration of cations (mono and divalent) and anions was determined using a Supermini X-Ray Fluorescence Spectrometer (Rigaku Corporation, Japan), using the semi-quantitative method for light elements analysis in helium atmosphere, following the manufacturer's protocol. Approximately 10 ml of surface water sample were weighted and then placed in the spectrometer. The percent of elements (cations and anions) in the amount of sample used in the apparatus after analysis was transformed into mg/ml. The UV-VIS investigations of the samples have been performed using a Nanodrop spectrophotometer. The absorbance and the protein content as mg/ml was determined at 280 nm.

2.3 Isolation of Halobacterial Strains

The samples have been taken in a one or five litter sterile bottles, in the summer or autumn as follows: the end of June 2013 (Amara sample 1) and the end of August 2013 (Amara sample 2); beginning of August 2013 – Ocnele Mari; beginning of September 2013 –from Balta Albă and Movila Miresei. The bottle was immersed approximately 15 - 20 cm into the water body at around 15 meters from the bank in case of Balta Albă, Movila Miresei and Amara and close to the bank in the case of Ocnele Mari. The bottle was sealed with screw cap after filling and maintained in appropriate conditions until to laboratory where the samples were transferred to 4^0C before chemical and microbiological analysis. The halophilic bacterial strains were isolated on JCM medium no. 168 which contained (g/l): Bacto casamino acids (5), Bacto yeast extract (5), sodium glutamate (1), trisodium citrate (3), $MgSO_4x7H_2O$ (29.5), KCl (2), NaCl (175.5), $FeCl_2x4H_2O$ (0.036), $MnCl_2x4H_2O$ (0.36 mg) and MH medium which contained (g/l): NaCl - 100, $MgCl_2x6H_2O$ - 7, $MgSO_4x7H_2O$ - 9.6, $CaCl_2x2H_2O$ - 0.36, KCl - 2, $NaHCO_3$ - 0.06, NaBr - 0.026, glucose - 1, proteose peptone - 5, yeast extract - 10 [24]. The medium pH was adjusted to 7.0 – 7.2 before autoclaving. One milliliter of the lake water sample was placed in a Petri dish and mixed with 30 ml of the autoclaved molten agar culture medium (cooled to 55-60ºC). After solidification, the plates were incubated at 37º C and 28º C for 7-10 days and at the end of this period the colony forming units (c.f.u.) number was counted [25].

2.4 Detection of Extracellular Hydrolytic Activities

The intensity of the enzymatic activities was evaluated based on the estimation of substrate consumption [26]. The following substrates were used, i.e. L-alanine-4-nitroanilide-hydrochloride for alanine-aminopeptidase (EC 3.2.1), 4-nitrophenyl-phosphate for alkaline phosphatase (EC 3.1.3.1), p-nitrophenyl-α-D-glucopyranoside for α-glucosidase (EC 3.2.1.20) and p-nitrophenyl- β -D-glucopyranoside for β –glucosidase (EC 3.2.1.21). The substrate solution (1 mg enzymatic substrate/1 mL NaCl 0,14M) was mixed with 1 mL water sample and the mixture was incubated for 6 h at 30°C. After incubation, the enzymatic reaction was stopped by adding 1 mL solution Na_2CO_3, 1M. The absorbance of the reaction product (p-nitrophenol or p-nitroanilline) was recorded spectrophotometrically (CECIL CE 1010 spectrophotometer) at 405 nm wavelength and its concentration was estimated by extrapolating the standard curve.

3. RESULTS AND DISCUSSION

The ionic composition in the investigated samples revealed that salinity is due to the presence of chloride and sulphur salts mainly with metal ions from alkaline and alkaline-earth groups. Potassium concentrations were detected from 0.5 mg/ml in Ocnele Mari brine to 12 mg/ml in Amara Lake. The silica was also present in all tested samples accordingly with the data showed in Table 1. On the other hand, sodium was absent in the sample from Amara and Balta Albă and present in concentrations of 18 mg/ml at Movila Miresei and 25 mg/ml in Ocnele Mari. The chloride content was varying from 13 mg/ml in Balta Albă, till 252 mg/ml in Ocnele Mari, respectively Table 1. Sulphur concentrations are 21 mg/ml in Amara Lake, 7 mg/ml in Balta Albă and Movila Miresei and traces in Ocnele Mari. Magnesium was present only in the samples from Amara and Ocnele Mari and was absent in other sites, but calcium was absent only in the samples from Balta Albă. In the other samples, the calcium content increased to 4 mg/ml (at Ocnele Mari). Traces of aluminum have been detected in the tested samples. Phosphorus and bromide has been detected only in Movila Miresei salt lake. Based on the results presented in Table 1 and accordingly to previously mentioned literature data, it could be argued that salt lakes Amara and Balta Albă should be called saline lakes and Movila Miresei and Ocnele Mari may be considered hypersaline water bodies.

The UV-VIS investigations of the tested samples showed that absorbance at 280 nm were 0.05 in the case of Ocnele Mari sample, very low, if compared to the value of 2.6 recorded in the case of Movila Miresei sample. For the lakes Balta Albă and Amara, the values are similar: 0.17 and 0.18 respectively. Following the absorbance values, the estimated protein content was 0.2 mg/ml in Amara salt lake, 0.16 mg/ml in Balta Albă salt lake, 3 mg/ml in hypersaline lake Movila Miresei and 0.05 mg/ml in the hypersaline body of water from Ocnele Mari Table 2. The ratio 260/280 nm showed a value of 0.7 in the case of hypersaline water sample from Ocnele Mari and a similar value, around 1.7 for Amara and Balta Albă salt lake. For the hypersaline lakes Movila Miresei, the recorded value has been 1.9. The UV-VIS values observed for Movila Miresei should be correlated with the color of the water. The lake appears to be characterized by a strong green color Fig. 1 which changes to yellow for human eyes perception related to the intensity of sun light. The color appears to be due to the alga belonging to *Bacillariophyceae* and *Chlorophyceae*. Generally, according to the data from the Tables 1 and 2, the values recorded in the case of UV-VIS investigation should be correlated either with the absence of sodium ions in the case of Amara and Balta Albă salt lakes, associated with a chloride content around 15 g/l and the presence of sodium ion and high values of the chloride content in hypersaline samples from Movila Miresei and Ocnele Mari, content which was 80 and 252 g/l respectively.

Table 1. The ionic composition (mg/ml) of the investigated surface water samples from saline and hypersaline lakes. "-"= the ion is absent. Excepting chloride content which are expressed in g/l, the other ions content is expressed in mg/ml

	Amara – sample 1	Amara – sample 2	Balta Albă	Movila Miresei	Ocnele Mari
Na	-	-	-	18	25
K	12	13	11	2	0.5
Mg	7	6	-	-	0.4
Ca	2	2	-	0.1	4
Sr	-	-	-	-	0.04
Al	-	0.1	0.3	0.1	0.2
Si	0.04	0.3	0.4	0.3	0.6
P	-	-	-	0.08	-
Br	-	-	-	0.5	-
S	21	21	7	7	0.4
Chloride (g/l)	17	15	13	80	252

Table 2. UV-VIS results in investigated saline and hypersaline environments

	Amara – sample 1	Amara – sample 2	Balta Albă	Movila Miresei	Ocnele Mari
Absorbance at 280 nm	0.18	0.18	0.17	2.6	0.05
Protein content (mg/ml)	0.2	0.2	0.16	3	0.05
Ratio 260/280 nm	1.6	1.7	1.65	1.9	0.7

The recorded physico-chemical data for the investigated samples Table 3 reveals that the pH values varied from 6.7 in the saturated brine from Ocnele Mari to 9.2 in Balta Albă salt lake. Based on the data showed in Table 1 and 3, it should be considered that Balta Albă salt lake harbors poliextremophilic conditions, namely high salinity and alkalinity. The density of the samples is correlated with the chloride content Table 1. and showed values around one in the case of saline samples and over one in the case of hypersaline samples. Other physical parameters of the samples, like temperature and conductivity are correlated with chloride content as resulted from the data showed in Tables 1 and 3.

Fig. 1. The particular color observed at Movila Miresei Hypersaline Lake. The physical aspect of the lake was recorded in photo images using a digital camera Canon PowerShot model Pro 1

Table 3. The general physico-chemical characteristics of investigated saline and hypersaline environments

	Amara – sample 1	Amara – sample 2	Balta Albă	Movila Miresei	Ocnele Mari
pH values	8.6	8.7	9.2	8.9	6.7
Chloride content (g/l)	17	15	13	80	252
Density (g/ml)	1.01	1.01	1.02	1.1	1.2
Conductivity (mS/cm)	35	40	48	265	416
Temperature (ºC)	26	30	26	25	24

The estimation of total colony forming units in tested samples revealed that they are inhabited by populations of halophilic bacteria and halophilic archaea. The registered numbers of colonies Table 4 are influenced by the culture medium used and decreased with the increase of the sodium chloride content. The colony forming units (c.f.u.) as showed in Table 4, varying from 45 in Ocnele Mari until to $33x10^2$ when using culture medium containing 200 g/l sodium chloride and from 69 until to $70x10^2$ in the context of 100 g/l sodium chloride in culture medium. Taking into account previously reported data fromvarious salted environments with similar salt content, the numbers appear to be low. For example, the Dead Sea and Great Salt Lake are inhabited by halophilic microorganisms of the magnitude of 10^6 cells/g, although fluctuating periodically [5,27] and Lake Chaka in northwestern China by 4.8 x 10^6 cells/ml [28]. On the other hand, the number of colonies is very close to those reported for several salt lakes from Romania [23], with chloride concentrations > 135 g/l. The data recorded in estimation of total c.f.u. number argued for the purpose of this study to evaluate extracellular hydrolytic activities related most probably to halophilic microorganisms. The extracellular hydrolytic activities are associated with the growth of microbial species (halobacteria and haloarchaea) which are involved in decomposition and degradation or organic matters in saline and hypersaline ecosystems. On the other hand, it should be noted that fungi belonging either to *Walemia* or *Basipetospora* genera have been isolated from all investigated samples.

Table 4. Determination of total colony forming units (c.f.u.) numbers in investigated surface water samples from saline and hypersaline investigated lakes; JCM and MH medium compositions are detailed in materials and methods section; "-" = no data available

	Amara – sample 1	Amara – sample 2	Balta Albă	Movila Miresei	Ocnele Mari
Culture medium JCM 168	22 $x10^2$	33 $x10^2$	47	58	45
Culture medium MH	-	-	$43x10^2$	70 $x10^2$	69
Presence of fungi	+	+	+	+	+

The extracellular hydrolytic activities revealed that α and β –glucosidase, alkaline phosphatase and aminopeptidase were present in the tested samples (Fig. 2). Generally, it should be noted that the enzymatic activity showed a similar profile with the chloride content, supported by UV-VIS investigations (Table 2).

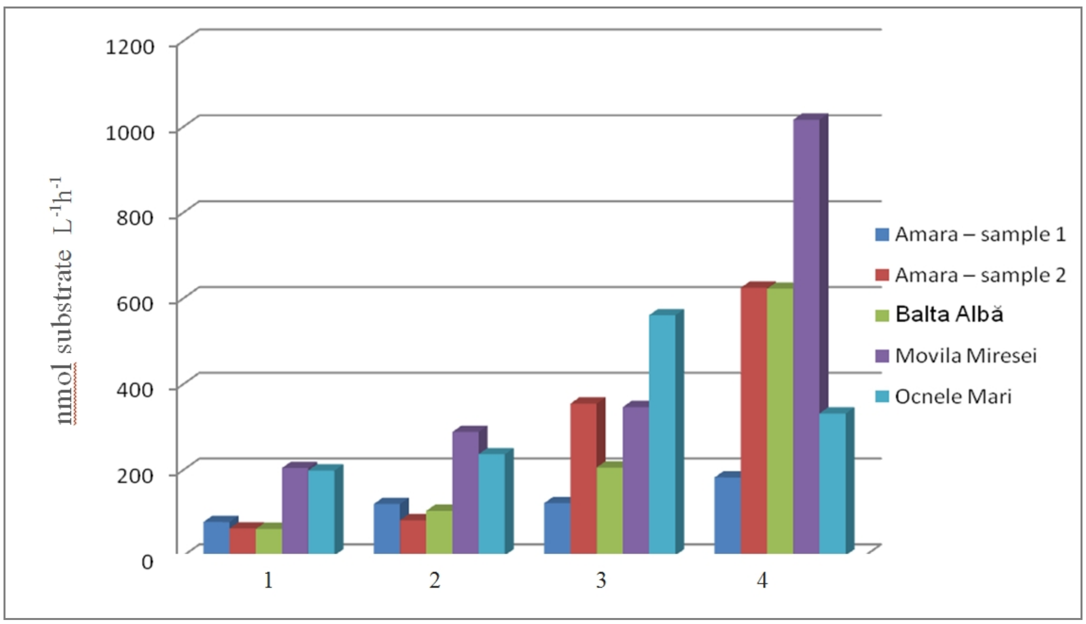

Fig. 2. The effect of physico-chemical parameters on the extracellular hydrolytic activities in investigated salt lakes; the values represented nmol substrate $L^{-1}h^{-1}$; 1 = α-glucosidase, 2 = β – glucosidase, 3 = alkaline phosphatase and 4 = aminopeptidase

Generally, the enzymatic activities involved in degrading and decomposition of sugar based substrates showed a low level of activity in saline waters where the sodium ion is absent, namely Amara and Balta Albă. The enzyme α –glucosidase showed high values in the hypersaline lakes Movila Miresei and Ocnele Mari and a similar profile was recorded also for β-glucosidase. A relatively small difference was observed for the activity of alkaline phosphatase. In this case, the enzymatic activity appears to be correlated in a direct proportion with the chloride content. The recorded data in the case of sample 2 from Amara Lake could be associated with the harvesting point or with the physico-chemical parameters of the sample Table 3. The enzyme aminopeptidase showed high values if compared with previously mentioned enzymes. In the case of Ocnele Mari brine, the values were lower than for with alkaline phosphatase. The recorded data presented in Fig. 2 supported that degradation and decomposition of protein macromolecules have higher values than sugar and phosphate based organic matter. According to the data from Table 4 and Fig. 2, itappears that the degradation and decomposition processes in the investigated saline and hypersaline environments were associated with the chloride content and the absence or presence of sodium. In this frame the recorded number of c.f.u. (JCM 168 culture medium) showed higher values in the saline investigated lakes, to $33x10^2$ in Amara salt lake and lower in the saturated brine from Ocnele Mari - 45 respectively, but values of aminopeptidase enzymatic activity are higher in the environments where the c.f.u. numbers was lower. A similar behavior was observed for all investigated enzymatic activities supporting the previous remarks that degradation and decomposition of organic matter in the investigated saline and hypersaline environments are associated with the chloride content Fig. 2 and Table 4.

4. CONCLUDING REMARKS

Accordingly to previous data, the pH values varying from 6.7 (Ocnele Mari Lake) to 9.2 in Balta Albă salt lake argued for extremophilic conditions. Following this data, the chloride content is 13 g/l in Balta Albă salt lake, around 15 – 17 g/l in Amara salt lake, 80 g/l in Movila Miresei hypersaline lake and close to saturation, 252 g/l respectively in the case of Ocnele Mari hypersaline sample. The chemical composition revealed that salinity is due to compounds of sulphur, chloride and potassium in lakes Balta Alba and Amara. In Movila Miresei, potassium is replaced by sodium and in Ocnele Mari the salinity is mainly due to chloride and sodium. Total c.f.u. numbers vary according to the culture medium used for isolation of viable halophilic bacterial and archaeal strains. Generally, the total c.f.u. number decreased with the increase of chloride content. Fungi are presented in all investigated salt environments.

5. CONCLUSION

Extracellular enzymatic activities revealed that decomposition processes of organic matters have a relatively high intensity in hypersaline samples, aminopeptidase activity having high level in Movila Miresei and alkaline phosphatase in Ocnele Mari. A similar behavior was noted for alpha and beta glucosidase but with slight differences between saline and hypersaline tested samples.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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