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EXTRACELLULAR HYDROLASES OF HALOPHILIC MICROORGANISMS ISOLATED FROM HYPERSALINE ENVIRONMENTS (SALT MINE AND SALT LAKES)

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Abstract

This work aims to reveal the ability of halophilic microorganisms, both bacteria and archaea, isolated from salted lakes and salt crystals from the salt mine, to produce a wide range of extracellular hydrolytic enzymes able to degrade several macromolecular substrates, such as sugar based polymers or proteins. A relatively wide positive spectrum of extracellular hydrolases for tested substrates was recorded from archaeal and bacterial strains isolated from investigated salted area. The number appears to be higher if comparing isolates from salt lakes with isolates from salt crystal. In the case of tested halophilic bacteria were found enzymes capable of hydrolyzing starch, casein, Tween80 and carboxymethylcellulose. In the case of microorganisms belonging to the Archaea domain, identified on the surface of the crystal and belonging to Halorubrum genus was detected the ability to degrade starch and seldom Tween80. In some cases, a combined hydrolytic activity has been observed. One halophilic bacterial strain combined cellulase and esterase activities and other strains combined two or more hydrolytic activities. The enzymes degrading starch appear to have a wide distribution and when compared the 16S rRNA phylogeny distribution of investigated strains with the absence or presence of amylase activity, the data showed a very high correlation degree.

Keywords: *extracellular hydrolases, halophilic archaea, halophilic microorganisms, salt lake, salt mine.*

INTRODUCTION

Halophilic microorganisms are widely distributed in environments characterized by salinity (mainly sodium chloride concentration) from negligible content until to saturation. Such kind of environments are largely spread in the entire world, the most frequently associated with hypersaline attribute being the Dead Sea and Great Salt Lake from United States (Minegishi, 2013; Oren, 2002; 2013). Excepting these areas, saline environments are also represented by salterns, salt mines, salted soil and many neutral or alkaline salted lakes like Wadi Natrum in Egypt (Oremland, 2013; Oren, 2013).

In Romania, several saline and hypersaline environments are represented by various anthropocentric and natural salt lakes, like Techirghiol, Balta Albă, Movila Miresei, Amara (Enache et al., 2012; Neagu et al., 2014) and several salt mines located mainly in the proximity of Carpathian Mountains or in sub-Carpathian hills. The present study has been focused on the salted area in Slănic Prahova where the exploitation of salt has been conducted from 1685 (Enache et al., 2008; 2009) until today following various technologies (Drăgănescu and Drăgănescu, 2001). The major consequence of salt exploitation for the environments in this area is represented by the formation of several salt lakes in the opening of former salt exploitations which are known as Green, Red and Shepherd Bath and Bride Cave. The previous studies (Enache et al., 2007; 2008) have indicated that the investigated areas were populated by halophilic microorganisms inhabiting both the surface and the inside of salt crystals (Enache and Kamekura, 2013). These organisms were both bacteria and archea belonging to the genera *Halobacterium*, *Halorubrum, Haloarcula,* predominantly identified within the crystal.

Generally, the hypersaline environments (including salt mine) represent an important source for isolating microorganisms that produce enzymes which could be regarded as

having industrial relevance (Moreno et al., 2013). Enzymes showing the activity over a wide spectrum of salinities have been isolated from various halophilic archaeal and bacterial microorganisms (Sanchez-Poro et al., 2003). These enzymes showed in many cases thermo- and alkalitolerant properties and those polyextremophilic features could be a tremendous advantage for their potential in various biotechnologies (Enache and Kamekura, 2010; Margesin and Schinner, 2001; Oren, 2010; Ventosa and Nieto, 1995) or in agriculture fields (Shivanad et al., 2012).
Extracellular hydrolases of halophilic microorganisms play a key role for these microbial strains to use organic compounds in salted environments and support their use in obtaining several products of commercial interest (Enache and Kamekura, 2010; Kamekura et al., 1982). This work aims to reveal the ability of halophilic microorganisms, both bacteria and archaea, isolated from salted lakes and salt crystals from the salt mine to produce a wide range of extracellular hydrolytic enzymes able to degrade several macromolecular substrates, such as sugar based polymers or proteins.

MATERIALS AND METHODS

The sample collection

The rock salt samples were taken from the wall of subterranean salt mine, Unirea, located in Slanic Prahova. Samples were taken from several points of the mine by courtesy of the mine staff. Crystals apparently (visually) considered to have biological material inclusion were used in further experiments as described below. Being estimated at the debut of the experiments that the probability to isolate halobacterial strains was very low, there were not tailored any statistical analysis. Accordingly, one gram of salt crystal with no apparent contamination by clay or soil was immersed and shaken in sterile 10% NaCl solution to wash the outside and was then dissolved in 50 ml of sterile 10% NaCl. In order to isolate halophilic bacteria, one ml of this solution was mixed with 20 ml of autoclaved molten agar medium (around 55^0C) MH with the following composition (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 (Ventosa et al.,1989). The halophilic archaeal strains were isolated from the crystal sample following previously described protocol (Enache and Kamekura, 2013).

The water samples were taken from the surface of salted lakes Shepherd Bath, Green Bath, Red Bath and Bride Cave located in Slănic, Prahova county, at about 100 km north from Bucharest, in the summer period. Approximately one liter of water from each site was taken in a sterile bottle, closed after filling and maintained in appropriate conditions until to laboratory where the samples were transferred to 4^0C before microbiological analysis. The archaeal strains were isolated in a medium (I) with the following composition (g/l): NaCl (125), $MgCl_2x6H_2O$ (160), K_2SO_4 (5), $CaCl_2x2H_2O$ (0.1), yeast extract (1), peptone (1), soluble starch (2), agar (20). The medium pH was 7.0-7.2. For enzyme detection experiments the strains were grown in 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). The medium pH was 7.0 – 7.2 before autoclaving.

Differentiation of archaea and bacteria isolates

The susceptibility of the novel isolates to antibiotics or bile salts, namely to chloramphenicol and sodium-deoxycholate was tested in order to confirm the belonging of the isolated strains to *Bacteria* domain and to differentiate them by halophilic archaea. For this purpose, there were used two solidified medium variants, with 0,004% deoxycholic acid sodium salt and with 0,002% chloramphenicol. Strains that were able to grow in the presence of Na deoxycholate were considered as halophilic bacteria (Kamekura et al., 1988; Kamekura and Seno, 1991).

Detection of extracellular hydrolytic activities

Biochemical test for starch hydrolysis was performed according to standard procedures at optimum concentration of NaCl for growth of

all the investigated strains. Hydrolysis of Tween 80 were detected using the method of Gutierrez and Gonzalez (1972). Casein and carboxymethyl cellulose hydrolysis was tested on solidified appropriate medium (JCM medium no. 168 for archaea and MH medium for bacteria) as previously described (Cojoc et al., 2009; Enache et al., 2008). All the investigation was performed in a medium supplemented with the appropriate substrate and using the optimum NaCl concentration for growth.

16S rRNA gene sequence analysis

Total DNA was extracted and purified using the method of Tamaoka adapted for halophilic archaea (Enache et al., 2007; 2008). The 16S rRNA genes were amplified by PCR, using the archaeal specific forward and reverse primers 5'-TCCGGTTGATCCTGCCG (position 8 – 24) and 5'-GGAGGTGATCCAGCCG (position 1540 – 1525), respectively. The resulted DNA fragments were sequenced using BigDye Terminator Cycle Sequencing Kit (Pharmacia Biotech) and ABI Prism DNA genetic analyzer (Applied Biosystems). The sequences obtained were analyzed using BLAST and aligned with other reported haloarchaeal 16S rRNA gene sequences using CLUSTAL W 1.7 software. A phylogenetic tree was reconstructed by the neighbor-joining method.

RESULTS AND DISCUSSIONS

The results obtained in this study and in previous works (Enache et al., 2008; Enache and Kamekura, 2013) revealed that investigated area hosts microbial strains belonging both to bacteria or archaea. In order to estimate the spectrum of extracellular hydrolases were investigated 16 archaeal strains and 13 bacterial strains isolated from salt crystal and 12 archaeal strains isolated from salted lakes. From the archeal strains originating from rock salt, six were isolated from the surface of the crystal and the remaining ten from the inside of the crystal (Enache and Kamekura, 2013).

The data showed in Table 1 and Figure 1 revealed that archaeal strains either from salt mine or salted lakes are devoid of capacity to degrade carboxymethyl-cellulose (CMC). Also, in the case of archaeal strains isolated from salt mine, the caseinolytic activity could not be detected (Table 1). The amylase activity was detected at 56% for archaeal strains isolated from salt mine and 83% at archaeal strains isolated from salted lakes. A different percentage was recorded for Tween 80 hydrolysis, namely 92% of the strains isolated from salted lakes harbored this activity by comparison with only 12% in the group of strains isolated from salt mine. This difference could be attributed to different anthropic factors which affected the investigated sites, the salted lakes and surface of the salt crystals from salt mine being exposed to high potential polluting factors which could generate substrates for esterase and lipases. This conclusion is sustained also by the high percent (85%) of bacterial strains isolated from salt mine (located at the surface of the crystal), which show the ability to degrade Tween80 (Figure 1, Table 1).

Table 1. Detection of extracellular enzymatic activities (positive/total tested strains) in cultivable halophilic archaea and bacteria isolated from Unirea salt mine and hypersaline lakes (Green Bath, Shepherd Bath, Red Bath and Bride Cave) in Slanic Prahova, Romania; CMC = carboxymethyl-cellulose

	Salt mine		Salted lakes
Tested substrates	Archaea	Bacteria	Archaea
Starch	9/16	3/13	10/12
Casein	0/16	6/13	2/12
Tween 80	2/16	11/13	11/12
CMC	0/16	2/13	0/12

Table 2. Distribution of tested cultivable halophilic archaea between genera of family *Halobacteriaceae*. Salted lakes: Green Bath, Shepherd Bath, Red Bath and Bride Cave. Salt mine: Unirea. All sampling sites are located in Slanic, Prahova, Romania

	Archaea	
	Salt mine	Salted lakes
Haloarcula	1	1
Halobacterium	2	0
Haloferax	0	11
Halorubrum	13	0
Total tested strains	16	12

This high percentage together with the facts that archaeal strains from salt mine which presented the capacity to degrade Tween 80 were located at the surface of the salt crystal and 92% of archaeal strains from salted lakes

were able of degrading Tween 80 support the hypothesis that positive strains from the salt mine, located either inside or on the surface of salt crystal, are transferred from other sites and should not be considered as native population of salt crystal. Around 15% of the bacterial strains isolated from salt mine showed ability to degrade carboxymethyl-cellulose (CMC) and 46% casein, in opposite with archaeal strains, where CMC degrading activity is absent and only 16% of strains isolated from salted lakes were able of hydrolyzing casein (Figure 1, Table 1). On the other hand, the amylase activity was less represented in the case of bacterial strains where only 23% showed this capacity (Table 1, Figure 1).

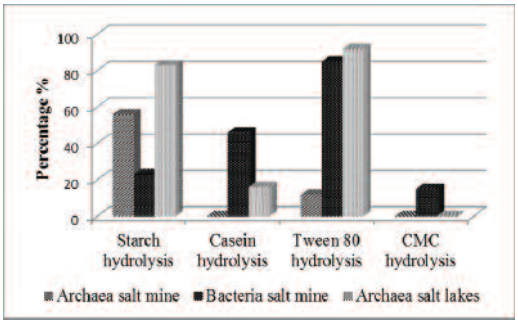

Figure 1. Distribution of extracellular hydrolytic activities in investigated sites

The 16S rDNA gene sequencing showed that investigated archaeal strains belonged to the genera *Haloarcula* (*Har.*), *Halobacteria* (*Hbt.*), *Haloferax* (*Hfx.*) and *Halorubrum* (*Hrr.*) (Figure 2, Table 2). The strains of *Har* genus could be isolated both from the salt mine and salted lakes but strains belonging to *Hbt* and *Hrr* genera could be isolated only from the salt mine. On the other hand, the strains belonging to *Hfx* genus were distributed only in salted lakes and could not be detected in salt mine (Tables 2 and 3).

Table 3. The distribution of tested cultivable halophilic archaea accordingly with sampling sites; the salted lakes and salt mines are described at previously tables

Lakes	Crystal of salt mine	
	Surface	Inside
Haloferax (predominant) *Halorubrum*	*Halorubrum*	*Halorubrum* (predominant) *Haloarcula* *Halobacterium noricense*

Based on this data could be concluded that in salted lakes are predominant *Hfx* genus and in salt mine *Hrr* genus. The phylogenetic tree reconstructed by the neighbor-joining method derived from sequences of 16S rDNA (Figure 2) revealed the same distance 0.041 which appears at the two nodal points on which are based the group of strains isolated from salt lakes (in blue color in Figure 2) and the group of strains isolated from salt crystal (red color marked strains isolated from the surface of the crystal and black color strains isolated from inside of the crystal).

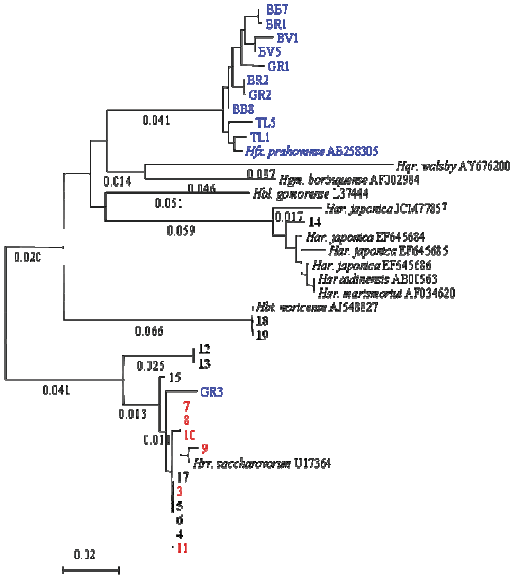

Figure 2. Phylogenetic tree reconstructed by the neighbor-joining method derived from sequences of 16S rDNA. The same distance 0.041 appears at the two nodal points on which are based the group of strains isolated from salt lakes (in blue) and the group of strains isolated from salt crystal (red color marked strains isolated from the surface of the crystal and black color strains isolated from inside of the crystal)

The clade of haloarchaea isolated from salt lakes is consituted by strains showing amylase and esterase activities (Figure 2, blue color clade). The clade of haloarchaea isolated from salt mine consists of strains characterized also by the presence of amylase activity (Figure 2, clade with red color). Some strains grouping separately, like GR3, 12, 13, 14, 18 and 19 are characterized by the absence of both amylase and esteraze activity. Based on the data showed in Figure 2 and Table 1 resulted that the presence of amylase activity is supported well

by phylogenetic classification, in spite of some incongruences recorded in the case of some strains isolated from salt mine. On the other hand the data showed in Table 1 revealed that some strains harbor more than one extracellular hydrolytic activity. The presence of such combined extracellular hydrolytic activities can offer a good potential for halophilic organisms to be applied as an economic alternative to some current biotechnological processes.

CONCLUDING REMARKS

A relatively wide positive spectrum of extracellular hydrolases for tested substrates was recorded from archaeal and bacterial strains isolated from investigated salted area. The number appears to be higher if comparing isolates from salt lakes with isolates from salt crystal and the enzymes degrading starch appear to have a wide distribution. When compared the phylogenetic distribution of investigated strains and the absence or presence of amylase activity, the data showed a very high correlation degree.

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REFERENCES

Cojoc R., Merciu S., Popescu G., Dumitru L, Kamekura M., Enache M., 2009. Extracellular hydrolytic enzymes of halophilic bacteria isolated from a subterranean rock salt crystal. Rom. Biotechnol. Lett., 14, 4658-4664.

Drăgănescu L., Drăgănescu S., 2001. The history of the evolution of salt working methods in Romania, from antiquity to the present. 17th Intl. Mining Congress and Exhibition of Turkey – IMCET, 627-633.

Enache M., Itoh T., Kamekura M., Teodosiu G., Dumitru L., 2007. *Haloferax prahovense* sp.nov., an extremely halophilic archaeons isolated from a Romanian salt lakes. Intl J Syst Evol Microbiol, 57, 393–397.

Enache M., Itoh T., Kamekura M., Popescu G., Dumitru L., 2008. Halophilic archaea isolated from man-made young (200 years) salt lakes in Slănic, Prahova, Romania. Cent. Eur. J. Biol., 3, 388-395.

Enache M., Popescu G., Dumitru L., Kamekura M., 2009. The effect of Na^+/Mg^{2+} ratio on the amylase activity of haloarchaea isolated from Techirghiol lake, Romania, a low salt environment. Proc. Rom. Acad., Series B, 1, 3-7.

Enache M., Kamekura M., 2010, The halophilic enzyme and their economical values. Rom. J. Biochem., 47, 47-59.

Enache M., Popescu G., Itoh T., Kamekura M., 2012. Halophilic microorganisms from man-made and natural hypersaline environments: physiology, ecology and biotechnological potential. In: Stan-Lotter H., Fendrihan S. (Eds.), Adaptation of Microbial Life to Environmental Extremes. Springer Wien New York, 173–197.

Enache M., Kamekura M., 2013. Halophilic archaea in the Neogene salt massif from Slănic Prahova, Romania. Oltenia. Studii şi Comunicări. Stiinţele Naturii, 29, 237–243.

Gutiérrez M.C., Gonzalez C., 1972. Method for simultaneous detection of proteinase and esterase activities in extremely halophilic bacteria. Appl. Microbiol., 24, 516-517.

Kamekura M., Hamakawa T., Onishi H., 1982. Application of halophilic nuclease H of *Micrococcus varians subsp. halophilus* to commercial production of flavoring agent 5'-GMP. Appl. Environ. Microbiol., 44, 994 – 995.

Kamekura M., Oesterhelt D., Wallace R., Anderson P., Kushner D.J., 1988. Lysis of halobacteria in bacto-peptone by bile acids. Appl. Environ. Microbiol., 54, 990-995.

Kamekura M., Seno Y., 1991. Lysis of halobacteria with bile acids and proteolytic enzyme of halophilic archaeobacteria. In: Rodriguez-Valera F. (Ed.), General and applied aspects of halophilic microorganisms. Plenum Press, New York, 359–365.

Margesin R., Schinner F., 2001. Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles, 5,73-83.

Minegishi H., 2013. Halophilic, acidophilic, and haloacidophilic prokaryotes. In: Seckbach J., Oren A., Stan-Lotter H. (Eds.), Polyextremophiles, Life under multiple forms of stress. Springer, Dordrecht Heidelberg New York London, 201-213.

Moreno M.L., Perez D., Garcia M.T., Mellado E., 2013. Halophilic bacteria as a source of novel hydrolytic enzymes. Life, 3, 38-51.

Neagu S., Enache M., Cojoc R., 2014. Extracellular hydrolytic activities of halophilic microorganisms isolated from Balta Albă salt lake. Rom. Biotechnol. Lett., 19, 1813-1820.

Oremland R.S., 2013. A random biogeochemical walk into three soda lakes of the western USA: with an introduction to a few of their microbial denizens. In: Seckbach J., Oren A., Stan-Lotter H. (Eds.), Polyextremophiles, Life under multiple forms of stress. Springer, Dordrecht Heidelberg New York London, 180-199.

Oren A., 2002. Halophilic microorganisms and their environments. Kluwer Academic, Dordrecht.

Oren A., 2010. Industrial and environmental applications of halophilic microorganisms. Environ. Technol., 31, 825-834.
Oren A., 2013. Two centuries of microbiological research in the Wadi Natrun, Egypt: a model system for the study of the ecology, physiology, and taxonomy of haloalkaliphilic microorganisms. In: Seckbach J., Oren A., Stan-Lotter H. (Eds.), Polyextremophiles, Life under multiple forms of stress. Springer, Dordrecht Heidelberg New York London, 103-119.
Sánchez-Porro C., Martín S., Mellado E., Ventosa A., 2003. Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. J. Appl. Microbiol., 94, 295-300.
Shivanad P., Mugeraya G., Kumar A., 2013. Utilization of renewable agricultural residues for the production of extracellular halostable cellulase from newly isolated *Halomonas* sp. strain PS47. Ann. Microbiol.
Ventosa A., Garcia M.T., Kamekura M., Onishi H., Ruiz-Berraquero F., 1989. *Bacillus halophilus* sp. nov., a new moderately halophilic *Bacillus* species. Syst. Appl. Microbiol., 12, 162–166.
Ventosa A., Nieto J.J., 1995. Biotechnological applications and potentialities of halophilic microorganisms. World J. Microbiol. Biotechnol., 11, 85-9