Halophilic microorganisms from Romanian saline environments as a source of extracellular enzymes with potential in agricultural economy

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HALOPHILIC MICROORGANISMS FROM ROMANIAN SALINE ENVIRONMENTS AS A SOURCE OF EXTRACELLULAR ENZYMES WITH POTENTIAL IN AGRICULTURAL ECONOMY

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Abstract: *Halophilic microorganisms, either bacteria or archaea, flourish in media with salinity levels varying from negligible until to saturation in NaCl and thus are considered extremophiles. Such kinds of media as salt lakes, salted soils, salt deposits or salt mines are widely distributed over the entire Romanian landscape. Several strains of halophilic bacteria and archaea have been isolated from such environments and characterized either by polyphasic taxonomy approach or by their ability to produce extracellular enzymes with putative potential for use in several domains like industry, agriculture and biotechnology. Even if sodium chloride is widely used in Romanian agriculture fields, little is known about salt microbiota, and its effect on the agricultural processes. This paper approaches the wide spectrum of extracellular hydrolytic enzymes with putative use in agriculture for treatment of polluted soils or recovery of salted soils. The strains isolated from inside of salt crystal are divided in two groups, one consisting of six strains belonging to Halorubrum, Haloarcula and Halobacterium genera and characterized by the absence of detectable extracellular enzymatic activity for the tested substrates and the other group consisting of four strains, all belonging to Halorubrum genus and characterized by the presence of amylolytic activity and the absence of other activities. The starch degrading enzymes appear to have a wide distribution both among the strains associated with salt lakes and among those isolated from rock salt.*

Key words: *halophilic enzyme, halophilic microorganisms, salted soil, salt*

INTRODUCTION

The soil represents the major factor for the agriculture even if is characterized by limited surface and shows fixity characters. In order to obtain a good agricultural production and interest products, mandatory compounds like fertilizers or other chemicals are introduced deliberately in soil. On the other hand the intensive mechanization and irrigation of agriculture could lead to an intensive exploitation for the agriculture and consequently, the production ratio and the obtained products could be affected either from the economical or the quality perspective. Following these processes, the already very slow action for recovering of soils by natural factors could be affected. Thus, it should be considered that the extracellular enzymes of halophilic microorganisms either bacteria or archaea could be used in this matter in order to facilitate the treatment of polluted soils or the recovery of salted soils.

The halophilic microorganisms are widely distributed in different saline and hypersaline environments. Generally, the saline water environments are differentiated in fresh water environments with an arbitrary concentration of dissolved salts higher than 3 g/L and hypersaline environments with total dissolved salts exceeding 35 g/L. When referring to saline environments, microbiologists denote a high interest for very well known salt lakes like Dead Sea or crystallizer ponds of salterns, environments almost saturated in sodium chloride, but also to salt mines or salt exploitation areas which harbor halophilic microorganisms (Enache et al., 2012).

These organisms grew optimally at high concentrations of salt and are generally dived in slight halophiles (optimum at 0.2 – 0.5M NaCl), moderately halophiles (0.5 – 2.5M NaCl) and extremely halophiles (over 2.0 M NaCl until to saturation). Halotolerant microorganisms are also able to grow until to 5M NaCl concentrations in culture media (Ventosa, 1989a). In opposition to halophilic bacteria, the halophilic archaea require at least 1.5M NaCl for growth and lyses in distilled water and hypotonic solutions like culture media without NaCl (Kamekura, 2007). Their first description in literature appeared in an old Chinese treatise on pharmacology written in the

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year 1592 (Kamekura, 2007) and also into the journal of Charles Darwin written in the year 1839, during the voyage of H.M.S. Beagle'. Nowadays are recorded more than 150 haloarchaeal species, distributed in 40 genera and isolated from the entire world (www.bacterio.net).

This paper focuses on the extracellular enzymes produced by several halophilic archaea and bacteria isolated from saline environments from Slanic Prahova, Romania, referring to their putative use in agriculture for treatment of polluted soils or recovery of salted soils.

MATERIALS AND METHODS

Sampling rock salt and salt water and isolation of halophilic bacteria

The rock salt samples were taken from the wall of subterranean salt mine, Unirea, located in Slanic Prahova. One gram of salt crystal with no apparent contamination by clay or soil was immersed and shaken in sterile 10% NaCl solution for five minutes to wash the outside and was then dissolved in 50 ml of sterile 10% NaCl. 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.,1989b).

The water samples were collected from various points of the lakes Green Bath, Red Bath, Shepherd Bath and Bride Cave. The 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. In some 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.

Detection of extracellular enzymatic activities

The MH medium without proteose peptone and glucose was designated as basal MH and used in the following experiments. The NaCl concentrations in the basal MH media varied until to 4M. This medium was used for halophilic bacteria. The JCM medium supplemented with appropriate substrates has been used for halophilic archaea. The basal medium was supplemented with different substrates acording with the experiments. The amylolytic, lipolytic (Tween 80 and olive oil), cellulase, gelatin and casein hydrolysing activities were tested following standard microbiological methods with some modification previously described (Cojoc et al., 2009).

RESULTS AND DISCUSSIONS

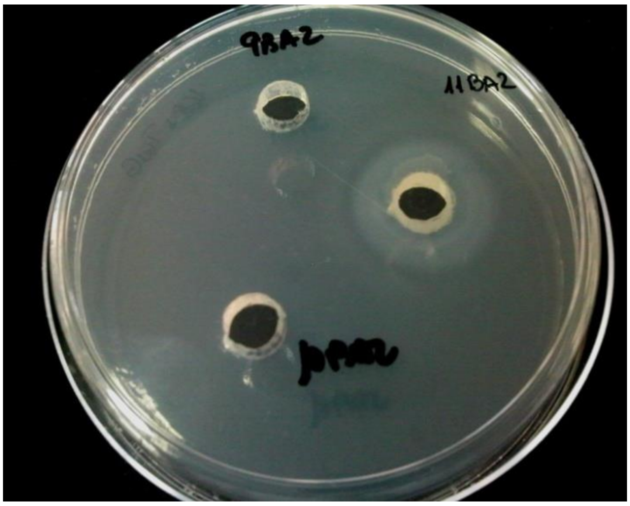
The obtained results revealed that both bacteria and archaea representatives inhabit salt waters and salt rock. In this last case, they could be isolated either from the surface of the crystal or from inside the crystal. Data from literature supported the identification of halophilic microorganisms in salt crystal (Dombrowski 1963; 1966; Stan-Lotter et al. 2000; McGenity et al., 2000). Previous investigations argued for the predominant presence of the *Haloferax* members in water samples taken from all salt lakes from Slanic Prahova area (Enache et al., 2008). These strains showed a relatively wide spectrum of extracellular hydrolytic enzymes. Enzymes active over a wide spectrum of salinities have been isolated from various halophilic archaea and bacteria microorganisms (Sanchez-Porro et al., 2003). These enzymes are often thermo- and alkalitolerant and those polyextremophilic features can be a tremendous advantage for their application in various biotechnologies (Margesin and Schinner, 2001; Oren, 2010; Ventosa and Nieto, 1995) or in agriculture fields (Shivanad et al., 2012).

The data from Table 1 confirm the presence of amylolytic activity at 10 investigated strains; the lipolytic activity has been detected at 11 strains; gelatin hydrolysis at five investigated strains and casein hydrolysis at two strains. The hydrolysis of Tween 80 by one of the investigated strains is also illustrated in Fig. 1, the strain surrounded by a halo resulted from the precipitation of reaction product being considered positive. The strain GR 3 assigned as *Haloarcula* sp. showed no hydrolytic activities. Excepting the strain Hfx 20 (*Haloferax* sp.), other remaining 10 strains showed combined hydrolytic activities which could be an advantage for their use in several biotechnological processes (Moreno et al., 2013; Sanchez-Porro et al., 2003) or for bioconversion of some agricultural products, agricultural waste and recovering of salted soils resulted from intensive agricultural exploitation.

Table 1 - Extracellular hydrolytic activities of halorchaeal strains isolated from salted lake in Slanic, Prahova; Hfx. = *Haloferax* species; Har. = *Haloarcula* sp.; 1 = Starch hydrolysis; 2 = Tween 80 hydrolysis; 3 = Gelatin hydrolysis; 4 = Casein hydrolysis; BR = Red Bath Lake; BV = Green Bath Lake; BB = Shepherd Bath Lake; GR = Bride Cave Lake (data from previous paper – Enache et. al. 2008)

	Hfx. BR1	Hfx. BR2	Hfx. BV1	Hfx. BV2	Hfx. BV5	Hfx. BB7	Hfx. BB8	Hfx. GR1	Hfx. GR2	Har. GR3	Hfx. 19	Hfx. 20
1	+	+	+	+	+	+	+	+	+	-	+	-
2	+	+	+	+	+	+	+	+	+	-	+	+
3	+	-	+	-	+	+	-	+	-	-	-	-
4	+	-	-	-	-	-	-	+	-	-	-	-

Fig. 1 - The Tween80 hydrolysis by halophilic bacteria isolated from a salt lake



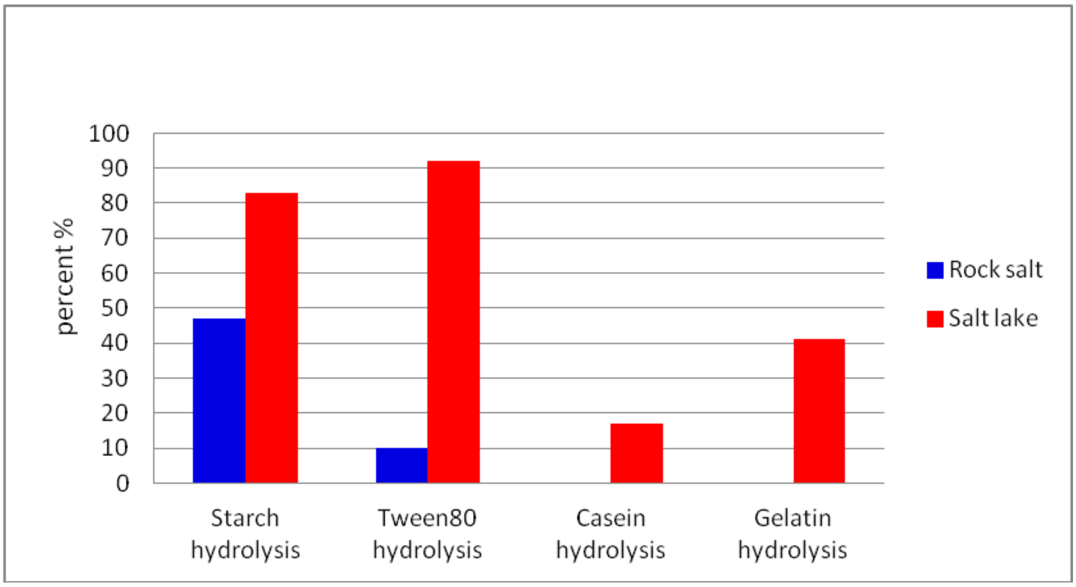
In a similar manner, the halophilic archaeal strains isolated from the surface or from the inside of the salt crystal showed the ability to hydrolyze starch and Tween 80, but were not able to degrade casein and carboxymethylcellulose (Table 2). Generally, the strains isolated from the surface of the crystal (table 2, numbers 3, 7, 8, 9, 10, 11) are characterized by the presence of amylolytic activity and the absence of other extracellular hydrolytic activities. The exception was the strain 8, which showed the capacity to degrade Tween 80 and strain 10, which was not able to hydrolyze any of the tested substrates.

On the other hand, the strains isolated from inside of salt crystal are divided in two groups, one consisting of the strains 13, 14, 15, 17, 18 and 19 and characterized by the absence of detectable extracellular enzymatic activity for the tested substrates and the other group consisting of the strains 4, 5, 6 and 13 and characterized by the presence of amylolytic activity and the absence of other activities. In this group, one exception was recorded for the strain 6 which showed also the capacity to degrade Tween 80 (Table 2). The first group covered six strains belonging to *Halorubrum*, *Haloarcula* and *Halobacterium* genera, and the second one covered four strains, all belonging to *Halorubrum* genus (Enache et al. 2008; Enache and Kamekura 2013).

Table 2 - Extracellular hydrolytic activities of halorchaeal strains isolated from salt crystal in Slanic, Prahova; *Halorubrum*: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17; *Haloarcula*: 14; *Halobacterium noricense*: 18, 19; A = Starch hydrolysis; B = Tween 80 hydrolysis; C = Casein hydrolysis; D = CMC hydrolysis; N = not determined

	Investigated strains																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	19
A	N	N	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-
B	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

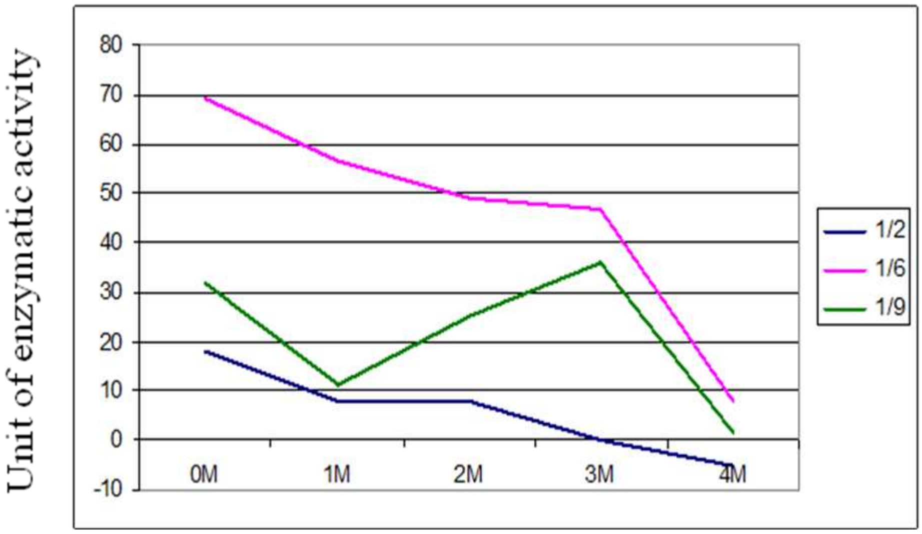
Fig. 2 - Presence of extracellular hydrolases among the investigated strains isolated from salt lakes and salt crystal, from Slănic Prahova area, Romania



When comparing the distribution of extracellular enzymatic activity between the strains isolated from salt water and salt crystal (Fig. 2), it resulted that the strains isolated from rock salt showed the capacity to degrade only starch and Tween 80, while the strains isolated from salt lakes were able to degrade all tested substrates. This way, it could be considered that the strains associated with rock salt are able to degrade compounds with α-1,4 and β-1,6 bonds which are characteristic for macromolecular compounds of sugar nature and also esteric bonds characteristic for the lipidic macromolecules. The strains isolated from the salt waters showed the capacity to degrade also the macromolecules of proteic nature. In this way they could be used more efficiently for the conversion of some agricultural products and the recovering of affected salted soils, taking into account the presence of combined extracellular hydrolytic activities (Table 1).

The data showed in figure 3 revealed the halotolerant behavior of extracellular amylase of some halophilic bacterial strains isolated also from salt rock. The activity of amylases was recorded until to 3.5M NaCl in culture medium, with the first maximum in the absence of sodium chloride and another maximum around 3M NaCl. We also consider that this behavior could be optimally exploited for several biotechnological processes or for bioconversion of some agricultural products, agricultural waste and the recovering of salted soils resulted from intensive agricultural exploitation.

Fig. 3 - The halotolerant behavior of amylases isolated from halophilic bacterial strains living on salt crystal; 1/2, 1/6 and 1/9 represent the investigated strains



CONCLUDING REMARKS

A relatively wide spectrum of extracellular hydrolases was recorded from archaeal and bacterial strains isolated from the investigated salted area. The number of the halophilic microorganisms which showed extracellular hydrolases appears to by higher in the case of strains isolated from salt lakes comparing with the isolates from salt crystal.

On the other hand, it should be noted that starch degrading enzymes appear to have a wide distribution both among the strains associated with salt lakes and among those isolated from rock salt. The halotolerant behavior of enzymes argues for their use in various agricultural fields, the recovery of some salted soils and for the bioconversion of some agricultural waste to useful products.

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