MUZEUL JUDEŢEAN ARGEŞ, PITEŞTI, ROMÂNIA
ARGESIS **- *STUDII ŞI COMUNICĂRI - seria ŞTIINŢELE NATURII,*** TOM XXI**, 2013**

EXTRACELLULAR PROTEOLYTIC ACTIVITY OF HALOPHILIC MICROORGANISMS ISOLATED FROM SALT ROCK

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ABSTRACT. Extracellular degrading enzymes are important to both pathogenic and saprophytic microorganisms to overcome host resistance and to utilize organic and inorganic materials in the environment. The main components in organic materials can be represented by proteins and enzymes must also exist for their breakdown in extreme environments such as subterranean salt deposits. The industrial and economic significance of proteolytic enzymes is very high and they can be used for bioconversion of some agricultural waste to useful products. This work focuses on the extracellular proteolytic activities of some halophilic microorganisms (mainly moderately halophilic bacteria) isolated from subterranean salt deposit formed in Neogene period in Slănic (Prahova) area. The obtained results revealed the presence of proteolityc activity which are influenced by NaCl concentration and composition of substrates used in the study.

Key words: proteases, halophilic microorganisms, haloarchaea, salt, Slănic Prahova.

REZUMAT. Activitatea proteolitică extracelulară a unor microorganisme halofile izolate dintr-un zăcământ subteran de sare. Enzimele extracelulare sunt importante atât pentru microorganismele patogene cât şi pentru cele saprofite întrucât conferă rezistenţă şi permit utilizarea compuşilor de natură organică şi anorganică prezenţi în mediul înconjurător. Materia organică este reprezentată în principal de proteine, iar enzimele necesare degradării lor trebuie să existe inclusiv în mediile extreme cum sunt zăcămintele subterane de sare. Importanţa industrială şi economică a enzimelor proteolitice este foarte ridicată, iar aceste enzime pot fi utilizate pentru bioconversia unor deşeuri agricole la produşi utili. Lucrarea de faţă are ca obiect activitatea proteolitică extracelulară a unor microorganisme halofile (în principal bacterii moderat halofile) izolate din zăcământul de sare de la Slănic Prahova format în perioada Neogenă. Datele obţinute dovedesc prezenţa activităţii proteolitice extracelulare precum şi dependenţa acesteia de anumite concentraţii de NaCl, dar şi influenţa ei asupra naturii substratului utilizat.

Cuvinte cheie: proteaze, microorganisme halofile, haloarhee, sare, Slănic Prahova.

INTRODUCTION

Halophilic microorganisms which need the constant presence of high salt concentrations for structural stability and viability can colonize a variety of environments like salt lakes (for example, the Dead Sea, the Great Salt Lake), crystallizer ponds of solar salterns, and salt mines around the world. In his paper from 1934, Baas Becking stated that the salt microorganisms are thus „a beautiful illustration of the principle that everything is everywhere", but with the amendment that „the environment selects" (Oren, 2011).

In Romania, the presence of halophilic microorganisms, both bacteria and archaea, was stated in a multitude of hypersaline environments (five salt lakes located in Prahova county and the Techirghiol Lake close to the Black Sea coast). For a few strains of halophilic archaea, preliminary taxonomic investigations have already been conducted (Enache et al., 2000; 2012), one strain being recently proposed as *Haloferax prahovense* (Enache et al., 2007). The almost 200 salt massifs located in the Romanian Carpathian area, by their favorable characteristics, such as surface proximity, superior purities of NaCl, or large reserves (for example, Cacica, Tg. Ocna and Ocnele Mari) constituted the subject of salt exploitation from antiquity until nowadays (Drăgănescu & Drăgănescu, 2001).

In the salt deposit from Slanic located in the outer Carpathian area, 45 km north of Ploieşti, underground of Prahova city, the salt extraction began in 1685 (Broşteanu, 1901) by the utilization of the bell type exploitation technology (Sencu, 1968). This salt deposit dating from the Neogen period is 2.8 km long, 0.8 km breadth and 45.5 m to 499 m thick (Drăgănescu, 1990), consisting of a mixture of grey and swarthy colored crystals, with smaller sizes than the white ones that can be found in other Romanian salt mines. The overall aspect of this deposit is variegated (Fig. 1), as a consequence of turnovers that took place during precipitation process, due to the climatic and sedimentary variations (Har et al., 2006). The sedimentary basin includes early formed authigenic minerals, of which halite is the most abundant and the last to precipitate in the basin, and also some allogenic minerals having the origin in the adjacent areas (Har et al., 2006). Extracellular degrading enzymes are important to both pathogenic and saprophytic microorganisms to overcome host resistance and to utilize organic and inorganic materials in the environment. Since the main component in organic materials can be represented by proteins, enzymes must exist for their breakdown also in extreme environments such subterranean salt deposits, previously thought to be inhospitable to life. Proteases play a key role in many biological processes and have numerous applications in biotechnology and industry.

Recent advances in the genetics, genomics and biochemistry of the halophilic microorganisms provide a tremendous opportunity for understanding proteases and their function in the context of a halophilic cell. Proteases participate in a number of biological processes such as the degradation of abnormal proteins, control of transcription factors, precursor processing, development and differentiation, regulation of the cell cycle and apoptosis (DeCastro et al., 2006).

The present paper focuses on the extracellular proteolytic activities of some halophilic microorganisms (mainly moderately halophilic bacteria) isolated from subterranean salt deposit formed in Neogene period in Slănic (Prahova) area. The researches of the area from microbiological point of view are highly supported by the economical interest based on the recreational and medical approaching of the salt lakes and salt mine in Slănic Prahova area. Consequently, the future investigations should be based also on the good co-operation with local factors directly involved in the economical exploitations of salt environments in order to manage a good plan for the future protection, conservation and sustainable development of the area.

MATERIALS AND METHODS

Sampling rock salt and isolation of halophilic bacteria.

The rock salt samples were taken from the wall of subterranean salt mine, Unirea, located in Slănic Prahova. The place from which the samples were taken is located at around 200 m below surface. The air temperature is 12 ^{0}C during the whole year, and the humidity is about 10% lower than at surface. Salt crystals were taken from the surface of the mine wall, around 2 m from the floor. 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 this process was repeated three times, and 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 ^{0}C) 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). After solidification, the Petri dishes were incubated for several days at 28 ^{0}C and colony forming units (c.f.u.) number was counted.

Estimation of percentages of proteinase positive strains in rock salt.

In order to estimate the percentage of strains possessing proteolytic activities, the salt solution was diluted serially with sterile 10% NaCl and spread on agar plate's containing casein. The colonies surrounded by a halo were considered positive. Numbers of total colonies and protease positive colonies were counted.

RESULTS AND DISCUSSION

Isolation of halophilic bacteria.

The investigated area appears to be a variegated salt deposit due to geological sedimentation along the times. The data showed in Fig. 1 revealed the stratification of salt massif following geological period and this data could be also related to the abundance of halophilic microorganisms, both bacteria and archaea in salted environments developed on the salt massif from Slănic, Prahova. Thus, a relatively high number of halophilic microorganisms populated

salt lakes and deposit from Slănic and some are isolated from inside of salt crystal (Enache & Kamekura, 2013) revealing that they could play an important role in biogeochemistry of the area.

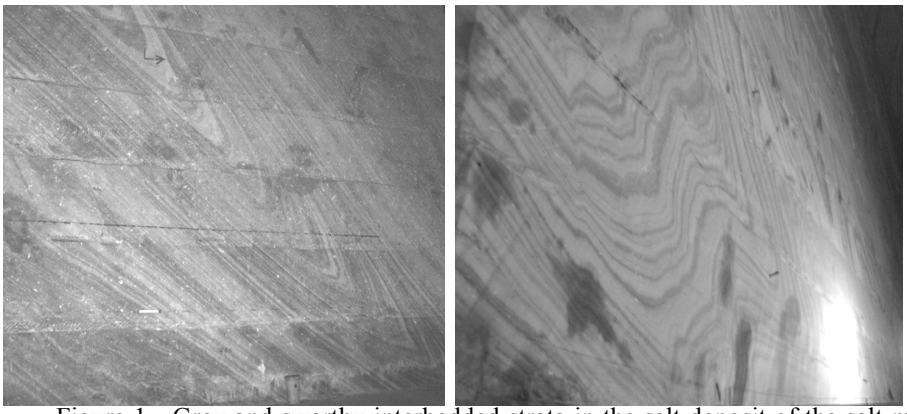


Figure 1 - Grey and swarthy interbedded strata in the salt deposit of the salt mine Unirea, Slănic Prahova.

The number of colonies obtained on the MH agar medium with 10% NaCl was calculated as around 3000 per gram of rock salt, as resulted from the data showed in the table 1. The number of colonies appears to be high if compared to previously reported data about total c.f.u. number from saline lakes in the area, but very close to the number of colonies recorded in Red Bath lake in Slănic area in the same year period and around three time lower than the number registered in same time period in Bride Cave salt lake, namely 750 c.f.u./ml (Enache et al., 2008a, b). The c.f.u. number in Red Bath was registered at around 2500 per mililiter (Enache et al., 2008a, b).

Estimation of percentages of proteinase positive strains in rock salt.

Following the purpose of the studies, the percentages of strains isolated from rock salt and having proteolytic activity were estimated. As resulted from the data showed in table 2 on agar plates with no NaCl added, around 13% of the colonies showed proteolytic activity.

On the other hand, at 1M NaCl, the number of positive proteolytic colonies remained relatively constant (Fig. 2; Tab. 2 and 3) in the diluted sample and decreased considerably in the native sample, respectively from 23 positive colonies recorded in the absence of NaCl to two colonies recorded in media with 1M NaCl, from 15% to 2% (Tab. 2) even if the total colony forming number appears to be similar (Tab. 2). The increasing of the salt content to 2M led to a high decreasing of total c.f.u numbers and to the absence of positive colonies for proteolytic activity (Tab. 2). These data argued that the proteolytic activity is affected by the increasing of the sodium chloride content, revealing its halotolerant behavior.

Table 1 - The number of c.f.u. estimated from rock salt dissolved in 10% sterile NaCl solution.

Sample	Absence of NaCl	1M NaCl	2M NaCl	
	48 h	48 h	48 h	72 h
Native sample	Over 800	700	0	Cannot read
Dilution 10^{-1}	50	43	2	5
Dilution 10^{-2}	6	11	0	0

Table 2 - Percentages of the proteolytic activity of samples from salt rock.

Sample	Absence of NaCl	1M NaCl	2M NaCl	
	48 h	48 h	48 h	72 h
Native sample	152 (23 positive) – 15%	133 (2 positive) – 2%	0	10
Dilution 10^{-1}	16 (2 positive) – 13%	31 (3 positive) – 10%	0	1
Dilution 10^{-2}	4	0	0	0

Taking into account the data presented in tables 2 and 3 resulted that after a period of incubation of four days the percent of colonies showing proteolytic activity remains relatively constant even if the total number of colony forming units increased considerably, namely from 133 to 520 in the native sample and from 31 to 44 in the diluted sample (Tab. 3).

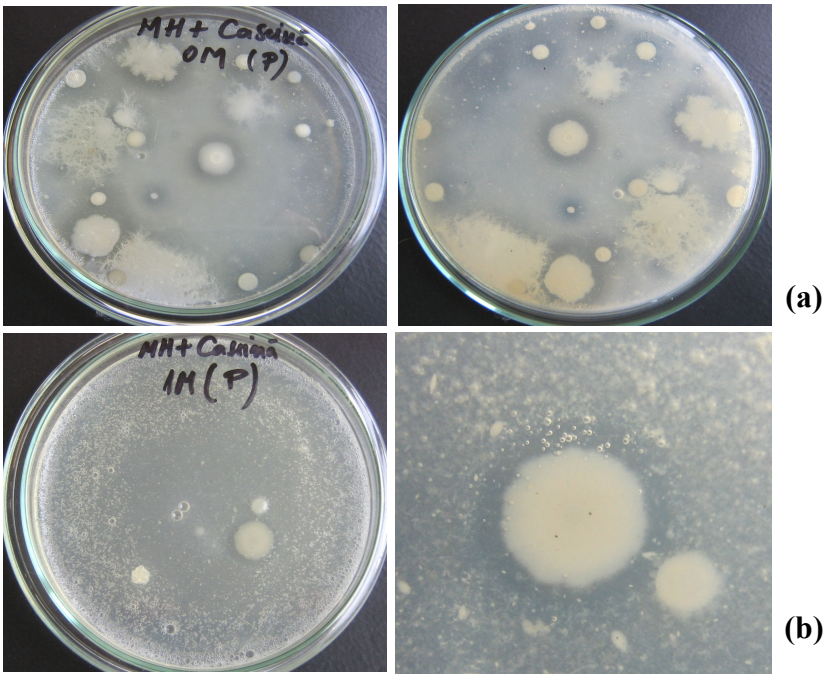
On the other hand, it could be observed that at the content of 2M sodium chloride in the cultivation medium there was not recorded any positive colony for the proteolytic activity. Also the total colony forming unit number showed a high decrease, from 520 recorded at 1M NaCl to 68 at 2M in the native sample. In a similar way in the diluted sample the number decreased from 44 at 1M NaCl to 12 at 2M NaCl.

Considering the data recorded in figure 2, some colonies appeared to be surrounded by halo with a relatively high diameter. These data argued either for the abundance of protein with enzymatic activity extracellularly secreted by halophilic bacterial cells or for a good catalytic activity of the enzymes which converted the substrate (casein) present in the cultivation medium.

Table 3 - Percentages of the proteolytic activity of sample from salt rock (recorded after 4 days).

Sample	Absence of NaCl	1 M NaCl	2 M NaCl
Native sample	Cannot read c.f.u. but halo can be observed	520 c.f.u. (around 10 surrounded by halo) – (2%)	68 c.f.u. (no halo was observed)
Dilution 10^{-1}	Cannot read c.f.u. but halo can be observed	44 c.f.u. (around six surrounded by halo) – (14%)	12 c.f.u. (no halo was observed)
Dilution 10^{-2}	Cannot read c.f.u. but halo can be observed	4 c.f.u. (no halo was observed)	1 – 2 c.f.u. (no halo was observed)

On the other hand, it could be observed that the diameter of the halo surrounding the colony judged as positive for proteolytic activity decreased at 2M NaCl content in culture media if compared with the halo diameter observed on media with 1M NaCl (Fig. 2a, b). This result supported the previous remark related to the halotolerant behavior of the enzyme with proteolytic activity detected in the investigated sample of salt crystal.



(a)

(b)

Figure 2 - Proteolytic activity of investigated halophilic bacteria on media without NaCl (a) or with 1M NaCl (b).

Since the area of microorganisms sampling is dedicated to a high touristic efflux, the registered data argued that the proteolytic activity of halophilic bacterial strains present in salt crystals is due to the abundance of proteinaceous substrates available following high human presence and impact. On the other hand, these preliminary investigations revealed the presence of proteolytic activity which is influenced by NaCl concentration and composition of substrates used in the study.

CONCLUSIONS

The study of proteases and their regulation in halophilic microorganisms continues to contribute to our overall understanding of the physiology of these unusual halophilic organisms that have adapted (evolved) their entire repertory of proteins to optimally grow in extreme environments. Future research in this field should be aimed at understanding how and when proteases function in the halophilic cell, revealing the mechanisms of targeting protein substrates for degradation by intracellular proteases, and identifying these protein targets (DeCastro et al., 2006).

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