Amylolytic activities excreted by the halophilic archaeon *Haloferax mediterranei* to assimilate available starch depend on the nitrogen source.

Abstract Several amylolytic activities have been isolated from controlled growing media containing starch and nitrate or ammonium acetate as carbon and energy source, excreted by the halophilic archaeon *Haloferax mediterranei*. These enzymes ~~found~~ produced in nitrate-containing medium were different from those produced by the organism when cultured in ammonium acetate-containing medium. *Haloferax mediterranei* was able to grow optimally in both media but not in a media with ammonium chloride and starch as exclusive nitrogen and carbon, respectively. Growth was significantly much lower when nitrate was replaced for ammonium, although there was significant amylolytic activity in the medium. At least six different activities were obtained in nitrate containing medium, but only five for ammonium containing one. These enzymes displayed different affinity for starch as chromatographic matrix, when eluted with maltose in a range from 0.02 M to 0.2 M maltose, and differ in their kinetic parameters for starch as substrate. The medium average length of the products obtained from cracking starch was different for each amylolytic activity, ranging from glucose to larger polysaccharides. Moreover, they exhibited different molecular masses, from 15 to 80 kDa. On the other hand, all of them behave as typical halophilic enzymes, requiring high salt concentrations from 2M to 4M NaCl for both stability and activity. Also it exhibited an optimal pH ranged from 7 to 8 and showed certain thermophilic behavior, with maximal activity at 50ºC to 60ºC. The study of the presence and behavior of this set of starch degrading enzymes will allow for a better understanding of how ~~our~~ halophilic organism obtains the adequate carbohydrates to be incorporated and optimally used.

Keywords Halophilic archaea · *Haloferax mediterranei* · Amylolytic enzymes · Isolation and biochemical characterization · carbon and nitrogen sources

Introduction

Starch represents a readily form to obtain the carbohydrates necessary to compete and succeed in the hard, extreme, media they have to endure. Organisms having the necessary machinery, both intracellular and extracellular enzymes, to hydrolyze them efficiently, are able to access to available glucose and a wide variety of oligosaccharides obtained from carbohydrate macromolecules (Vihinen and P. Mäntsälä 1989, Bonete el al 2007).

Due to the complex structure of starch, its digestion occurs in several stages in a great variety of organisms including ourselves, humans (Groot et al 1989). Initially amylases provide a partial digestion, which breaks down polymeric starch into shorter oligomers, and this partially digested starch is then extensively hydrolyzed into smaller oligosaccharides by other glycosylases. The enzymes responsible for these transformations belong, most of them, to α-amylase family. According to their role, we may classify them as endoamylases, exoamylases, debranching enzymes and transferases (van der Maarel et al 2002). Endoamylase cleave α-1,4-glycosidic bonds in the inner part of a chain, such as α-amylase (EC 3.2.1.1), that yields oligosaccharides with a α-configuration and α-limit dextrins. Exoamylases cleave α-1,4 glycosidic bonds in the external part of starch, such as β-amylase (EC3.2.1.2), yielding maltose and β-limit dextrin; or both α-1,4 and α-1,6 glycosidic bonds, as glucoamylase (EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20) producing only glucose. Debranching enzymes such as isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41), only hydrolyze α-1,6 glycosidic to yield maltotriose and linear oligosaccharides. Finally, transferases cleave an α-1,4 glycosidic bond of a donor molecule and transfer this cut part to another glycosidic acceptor. Amylomaltase (EC 2.4.1.25) and Cyclodextrin glycosyltransferase (EC 2.4.1.19) are transferases that create a new α-1,4 glycosidic bond while branching enzyme (EC 2.4.1.18) forms a new α-1,6 glycosidic bond. Cyclodextrin glycosyltransferase produces a series of non-reducing cyclic dextrins, α−, β- and γ-cyclodextrins (Bonete et al 2007).

Our halophilic archaeon has already been reported to produce two extracellular starch degrading enzymes when it was grown in ammonium acetate medium: α-amylase (Pérez-Pomares et al. 2003) and cyclodextrin glycosyltransferase (Bautista et al. 2012). This glycosyltransferase may act degrading and transforming starch in several ways, including both coupling and disproportionation activities, but cyclization was, by far, its main activity, yielding a mixture of cyclodextrins. Its sequence revealed an open reading frame of 2142 bp, corresponding to a protein of 713 amino acids, with high homology with those belonging to the α-amylase family, and was secreted to the extracellular medium by the Tat pathway (Bautista et al 2012). On the other hand, the α-amylase already reported produced a mixture of different dextrins and maltose. Its behavior is very similar to the amylases from *Halobacterium salinarum* (Good and Hartman 1970), and *Natronococcus amylolyticus* (koboyashi et al. 1992) and to that from the moderately halophilic bacteria *Halomonas meridiana* (Coronado el al 2000). Besides, grown in a starch containing medium, also produces three cytoplasmic activities (AMY1, AMY2, and AMY3), all of them typically halophilic, and reported to produce mainly small oligosaccharides from starch or dextrins. All these enzymes appeared when the organism grew in media with ammonium acetate and starch, except

AMY2, detected when the organism was in a medium with glycerol as carbon source (Pérez-Pomares et al 2009). The degradation of starch in the adequate way, previous to its assimilation appears to be essential for its optimal use. The aim of this study of the implied enzymes was a deeper understanding of how the organism succeed in getting profit of the starch, in different conditions of growth, with different nitrogen sources.

Materials and methods

Growth conditions and crude enzyme preparation

H. mediterranei strain R4 (ATCC 33500) (Rodríguez-Valera et al. 1983) was grown in 25% (w/v) salts, at 37ºC, pH 7.2 and supplemented with different carbon and nitrogen sources. ~~We used t~~Three different media was used, named as A, B and C. Medium A contained 0.1M potassium nitrate and starch 0.2% (w/v), as nitrogen and carbon sources, respectively; medium B: 1% ammonium chloride and starch 0.2% (w/v); and medium C, 1% (w/v) ammonium acetate, source of both carbon and nitrogen, and supplemented with 0.2% (w/v) soluble starch (adding a previously filter-sterilized starch stock solution). The clarified media used for further assays was obtained by harvesting cells by centrifugation at 10,000 rpm for 30 min at 4ºC, at least two times, until no suspension of particles was observed.

Determination of degrading activity

The activities were routinely assayed by the iodine binding assay, in 20 mM Tris–HCl buffer pH 7.5, at 40ºC, 3 M NaCl (activity buffer). We adjusted the adequate starch concentration in the reaction mixture with potato-soluble starch (Sigma) and terminated it by cooling in ice. Color appeared by the addition of iodine solution [4% potassium iodide (w/v), 1.25% iodine (w/v)] and the loss of starch was determined spectrophotometrically at 600 nm.

One unit of activity was the amount of protein that hydrolyzed 1 mg of starch in 1 min (Haseltine et al. 1996).

We measured also the activity by the dinitrosalicylic acid method (Bernfeld 1955) in order to determine the release of reducing end sugars.

The reaction was kept at the temperatures tested in a thermostatic bath and stopped in ice. The dinitrosalicylic acid reagent (100 µL) was added to the samples and this mixture was heated at 100ºC in a boiling bath for 10 min. We followed the development of color spectrophotometrically at 540 nm. One unit of activity was defined as the amount of protein, which produced 1 µmol of

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reducing ends in 1 min. Maltose was used ~~to produce~~for a standard curve. We performed all assays, at least, in duplicate and average values obtained.

Cyclodextrin glycosyltransferase activity assay

The cyclization activity was determined ~~by~~ using different dyes: methyl orange, phenolphthalein and bromocresol green~~, respectively~~. The production of cyclodextrins was analyzed spectrophotometrically by the absorbance decreasing at 490 nm in the case of a-CD and 552 nm for b-CD, and by the increase in absorbance at 630 nm for c-CD. The reaction mixture contained potato starch solution 1% (w/v) in 0.1 M Bis-Tris propane, pH 7.0, 1.5 M NaCl buffer (buffer C). One unit of cyclization activity (U) is defined as the amount of enzyme that produces 1 lmol of a-, b- or c-CD, as described by Bautista et al. (2012).

Enzymes isolation

The supernatant from 100 mL of culture was passed through a starch column prepared with insoluble starch packed in a 2.5 9 10 cm column. This column was intensively washed with 3 M NaCl 0.02 M Tris–HCl pH 8.0 buffer, as previously described in Perez-Pomares et al (2009). The pass through was collected and the column intensively washed in 20 mM Tris-HCl buffer 3M NaCl (buffer A) until no activity at all was observed in the fractions obtained. The elution of the proteins retained in the column was performed by using an increasing concentration of maltose, with a gradient of maltose from 0 to 0.2 M for both media. We used a discontinuous gradient of crescent concentrations of maltose for better isolation of the different enzymes and used for further studies. We tested Amylase activity in all the fractions and pooled the more active, previously to apply them to a Sephacryl S-300 and a Sepharose 4-B gel filtration column that also served to determine its molecular weight. We determined the protein concentration by the Bradford method (Bradford 1976).

The active fractions were concentrated and desalted to a final volume of 500 µL by ultrafiltration in an Amicon concentrating unit using a 10,000-MW cut-off membrane, and the enzyme was analyzed by SDS-PAGE for purity and subunit molecular weight.

Analysis of reaction products

Thin-layer chromatography (TLC) was used in order to analyze the products of the different amylolytic activities. We incubate each purified enzyme overnight, as described previously, and spotted in silica gel plates. We developed each plate with solvent mixtures containing different proportions of isopropanol–

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ethyl acetate–water. Standard 50 mM or 1% solutions of the carbohydrates used as substrates were also included in the plates.

The oligosaccharides were detected by spraying 1% diphenylamine, solved in acetone containing 10% phosphoric acid to the TLC plate, and heated at 160ºC for 10 min as described by Kobayshi et al. (2000).

Besides, the hydrolysis products of the amylolytic enzymes were analyzed by gel filtration in a 2.5 x 30 cm Sephadex G-100 column. The lower molecular weight products where further analyzed by high-performance liquid chromatography (HPLC) in a carbohydrate column (4.6·250 mm Waters, Milford, Mass.) in 50 mM phosphate buffer pH 7, 2 M NaCl. The standards ~~were~~ consisted of maltose, maltotrioside, and maltohexaoxide prepared in concentrations of 1 mM in the same conditions as the reaction products.

Effect of salt concentration, pH, and temperature

We tested the effect of salt concentration on enzyme activity by measuring the activity at 40 °C in 20 mM Tris-HCl, pH7.3, buffers containing different NaCl concentrations. For each salt concentration, starch concentration varied from 0.02 to 0.2% (w/v).

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The stability of the amylase at different salt concentrations (0 M, 2 M and 3M) was determined by incubating the enzyme in buffers containing the studied salt concentration and measuring the activity of aliquots at different times.

For pH studies different buffers were used: 0.2 M citric acid/ phosphate for pHs from 4.5 to 7, 0.2 M Tris-HCl buffers for pHs from 7 to 9, and 0.2 M CHES buffers for pHs 9 to 10. All of them contained 3 M NaCl. The pH checked after each reaction, showed no changes with respect to the initial values. The assays to study the dependence of temperature were carried out in 0.2 M phosphate buffer, pH 7.5, 3 M NaCl, at different temperatures. For each pH and for each temperature, starch concentrations varied from 0.5 to 5 mg/mL.

Inhibition by maltose

We tested the inhibition by maltose by measuring the activity at 40 °C in 20 mM Tris-HCl, pH7.3, buffers containing different NaCl concentrations. For each maltose concentration, starch concentration was varied from 0.67 mg/mL to 6.7 mg/mL of starch; and maltose concentrations tested were; 0, 0.075M, 0.15, 0.25 and 0.35 M.

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Data processing

Reciprocal initial velocities were plotted versus reciprocal substrate concentrations. All plots were linear. Initial velocities (v) obtained at each salt concentration, pH, or temperature, respectively, by varying the substrate concentration (S), were fitted to Michaelis-Menten equation to obtain maximum velocity (V), the Michaelis constant (K) for the substrate, and the apparent first-order constant for the interaction of enzyme and substrate (V/K). SigmaPlot program (Jandel Scientific, v. 1.02) was used, applied the algorithm of Marquardt-Levenberg. Same processing was followed with data from each salt concentration and for each maltose concentration. In the study of the inhibition of the enzyme with maltose, the inhibition constant was determined adjusting data to the competitive inhibition reaction.

Data from the stability studies were fitted as a logarithm of the residual activity versus time for each salt concentration, pH, or temperature studied.
The half-life and the pseudo-first-order constant for the denaturing process were determined from the slope of the straight lines obtained.

Peptide sequencing by mass spectrometry (ESI-MS/MS)

Coomassie-stained protein bands were excised from the gel, in- gel digested with trypsin (sequencing grade porcine trypsin, Pro- mega), according to the University of Alicante Mass Spectrometry Facility in-gel digestion procedure, and subjected to ESI-MS/ MS. Analysis was performed in a Q-Tof (Micromass) coupled to a CapLC (Waters) chromatographic system. The tryptic peptides were purified using a Waters Opti-Pak C18 trap column. The trapped peptides were eluted using a water/acetonitrile 0.1% (v/ v) formic acid gradient and separated by a 75 mL internal diameter, capillary column home-pack with C18 silica. Data were acquired in data- dependent mode, and multiplied charged ions were subjected to MS/MS experiments. The MS/MS spectra were processed using MAXENT 3 (Micromass), and manually sequenced using the PEPSEQ program (Micromass). The primary sequence was analyzed using the BLAST database (http://www.ncbi.nih.gov/BLAST)

Results

Amylolytic activities excreted into different media

For each media, containing nitrate, A, ammonium chloride, B, and ammonium acetate, C, we obtained different total starch degrading activity, which can be due to the amount, the concentration, of the enzymes excreted or also to the different set of enzymes secreted. However, nearly no growth was observed in the ammonium chloride containing Medium B. The maximal weight of cells

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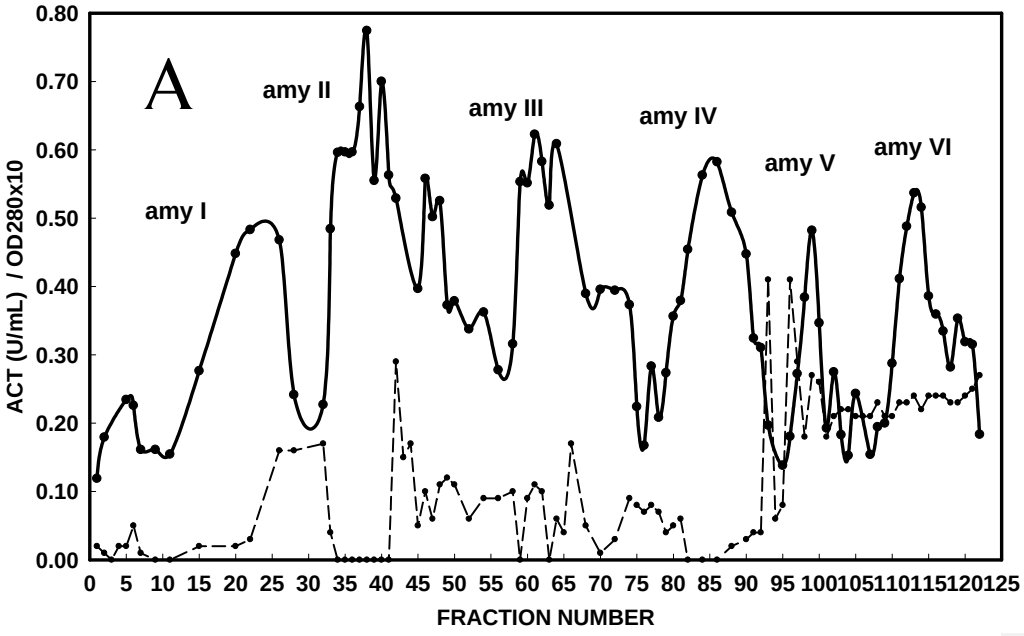
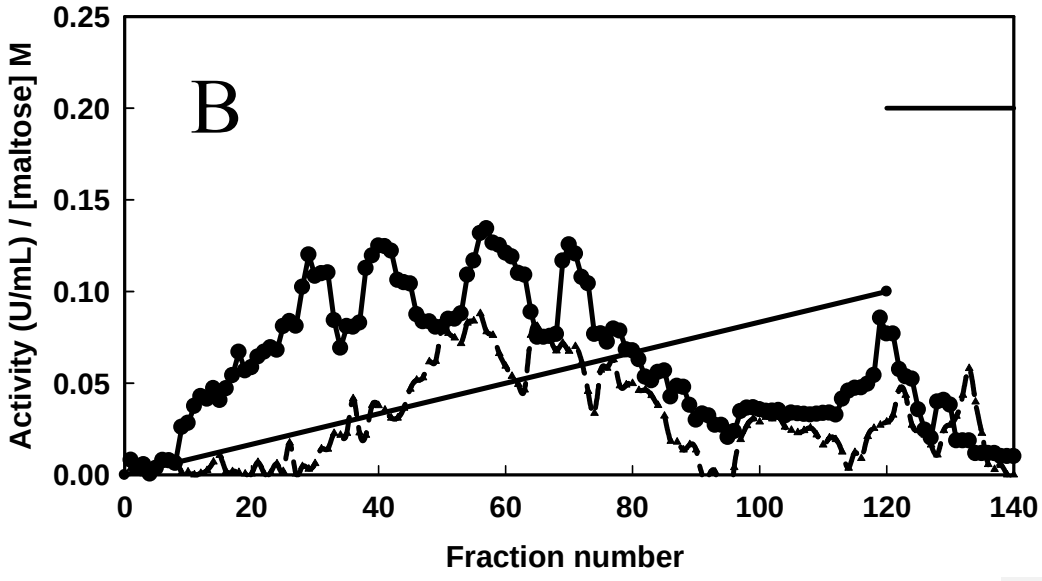
grown in medium A was 2.5 ± 0.5 g wet cells per 100 mL culture, similar to that found in medium C, 2.2 ± 0.5 g, and much higher than that found for medium B 0.5 ± 0.15 g per 100mL culture. However, in spite of this residual growth, we detected amylolytic activities in it. These activities, compared with that found in Medium A using a starch column, as previously described, with a gradient from 0 to 0.2 M maltose, are displayed in Figure 1A.

Figure 1A. Chromatography of clarified external medium from a culture of *H. mediterranei* in medium A, performed in a small column filled with insoluble starch. Proteins retained were eluted with buffer A, containing increasing concentrations of maltose: 5, 10, 20, 50, 75, 100 and 200 mM maltose, which corresponds to the fractions from 1 to 29, 30 to 54, 55 to 74, 75 to 95, 96 to 108 and from 109 to 125, respectively. The dashed line represented the protein content, as absorbance at 280nm.

Figure 1B. Amylolytic activities isolated as in figure 1A, but using a linear gradient from 0 to 0.1M maltose and a final elution with 0.2M maltose. The circles (●) united with a continuous line corresponds to clarified external medium of cells grown with potassium nitrate as nitrogen source and the up triangles(▲) and dashed line, the same but with ammonium chloride as nitrogen source. For all the fractions collected, the volume was 3 mL.





Medium C, was used exclusively as growth, and amylolytic activity control, since previous studies had already been reported, including the fully

characterization of two external amylolytic enzymes: α-amylase (Pérez-Pomares et al 2003) and cyclodextrin glycosyltransferase (Bautista el al 2012). Moreover, medium C contains no one but two different carbon sources (acetate and starch). In order to study specifically the exclusive use of starch as carbon source, the present study focused on the starch degrading enzymes excreted in media A and B, where the set of enzymes permit full usage of starch as exclusive carbon source.

The results in Figure ~~1~~ 1 B display again a set of different amylolytic activities excreted. There were at least six main activities named as amyI, to amyVI. The little peak at approximately 5 mM maltose was not marked. The complexity in number of the enzymes implied leaded us to pay attention only to the more prominent activities. Growth of the organism in ammonium chloride produced in general enzymes that eluted at higher maltose concentration, producing practically undetected activity at concentration higher than 0.2M maltose. The chromatography produced partially purified samples, as stated in Table 1, with a high purification factor, and further purified as previously described. The last purification step also served to estimate their molecular weight.

Table 1. Purification of the excreted external amylolytic activites from *H. mediterranei* grown in a medium containing 0.1 M potassium nitrate and 0.2% starch. Yield values were estimated considering that a volume of 3mL of sample from starch column was applied to Sephacryl S300.

		Volume (ml)	Activity (U/ml)	yield(%)	protein conc (mg/ml)	specific activity (U/mg)	Purification factor
clarified external nedium		100	1.8	100	0,25	7.2	1
amyI	starch collumn	21	0.42	4,9	$41 \cdot 10^{-3}$	10.2	1.4
	Sephacryl-S300	14	0,035	39	$1.0 \cdot 10^{-3}$	35	4,9
amyII	starch collumn	18	1.10	11	$29 \cdot 10^{-3}$	37.9	5.3
	Sephacryl-S300	6	0.086	28	$2.0 \cdot 10^{-3}$	43	6.0
amyIII	starch collumn	9	0.80	4	$19 \cdot 10^{-3}$	42.1	5.8
	Sephacryl-S300	16	0.11	73	$1.8 \cdot 10^{-3}$	61.1	8.5
amyIV	starch collumn	18	0.99	9.9	$16 \cdot 10^{-3}$	61.9	8.6

	Sephacryl-S300	12	0.12	48	$0{,}83 \cdot 10^{-3}$	144.5	20
amyV	starch collumn	15	0.84	7	$61 \cdot 10^{-3}$	13.8	1.9
	Sephacryl-S300	6	0.114	27	$5.0 \cdot 10^{-3}$	22.8	3.2
amyVI	starch collumn	15	0.78	4,9	0.09	8.6	1.2
	Sephacryl-S300	10	0.07	30	$7.2 \cdot 10^{-3}$	9.7	1.4

However, the molecular mases obtained, summarized in Table 2, depended on the matrix gel, dextrose gels Sephacryl S300 and Sepharose 4B, used, and so we should consider them only as estimated masses. The chemical composition of the chromatographic polymer used, its carbohydrate related nature, may be leading, in general, to affinity interactions enzyme-bed polymer, and consequently, to underestimated molecular weights, lower than those obtained by SDS-PAGE analysis (Figure 7). Attending to these values in table2, and position of the main bands in Figure 7, corresponding to the amylolytic enzymes, we should consider monomeric enzymes all of them.

Table 2. Estimated molecular weights for the excreted amylase activities by *H. mediterranei*, by gel filtration in both, Sephacryl S300 and sepharose 4B.

	Sephacryl S300	Sepharose 4B	average Mr
AMY1	27 ± 4 kDa	40 ± 9 kDa	34 ± 7 kDa
AMY2	19 ± 3 kDa	29 ± 7 kDa	24 ± 5 kDa
AMY3	23 ± 4 kDa	20 ± 6 kDa	22 ± 5 kDa
AMY4	16 ± 3 kDa	43 ± 9 kDa	30 ± 6 kDa
AMY5	19 ± 3 kDa	43 ± 9 kDa	31 ± 6 kDa
AMY6	16 ± 3 kDa	17 ± 5 kDa	17 ± 4 kDa

Once purified, we used these purified samples in the further studies.

Maltose inhibition and kinetic parameters

Data for the interactions between maltose and the starch degrading enzymes displayed competitive patterns for Lineweaver-Burk plot, all of them similar to that shown in Figure 2 for amyII.

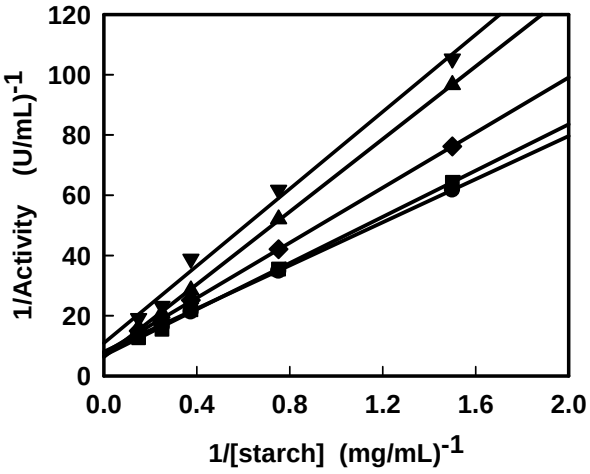


Figure II. Maltose inhibition pattern obtained for the hydrolysis of starch by amyII. Symbols (●) 0M maltose; (■) 0.075M maltose; (♦) 0.15M maltose; (▲) 0.25M maltose; (▼) 0.35M maltose

~~In~~ Table 2 ~~are~~ summarized the kinetic parameters from the equation for competitive inhibition. The Michaelis-Menten parameters, calculated by fitting data to inhibition equations: V and K, were very similar for all activities. However, maltose did not exhibit any significant inhibitory effect for amyI nor amyV; meanwhile, in the conditions tested, the other activities had a very similar behavior, with Ki values in the same range (values, from 360 to 530 mM, higher than the maltose concentration necessary to completely elute the starch hydrolases from the column). Consequently, we did not necessarily relate maltose interactions with starch and/or enzymes, to the catalytic core of these enzymes. Moreover, the different behavior of these activities regarding inhibition by maltose was indicative of concurrence of several enzymes implicated in starch degradation and processing.

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Table 3. Kinetic parameters and the inhibition constant Ki for maltose as competitive inhibitor of the hydrolysis of starch catalyzed by amyI, II, III, IV, V and VI.

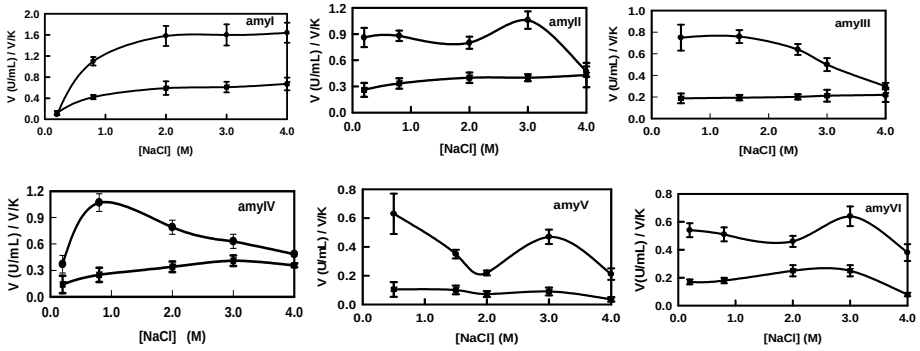
Kinetic parameters	V (U/mL)	K (mg/mL)	Ki mM
amyI	0.75± 0.14	5,3± 1.7	-
amyII	1.26 ± 0.10	4.1 ± 0.7	360 ± 7
amyIII	0.98 ± 0.10	3.3 ± 0.8	470 ± 16
amyIV	1.7 ± 0.3	5.0 ± 1.5	530 ± 19

amyV	0.47± 0.05	5.2± 1.1	-
amyVI	0.82 ± 0.08	3.9 ± 0.8	357 ± 9

Salt, pH and temperature requirements

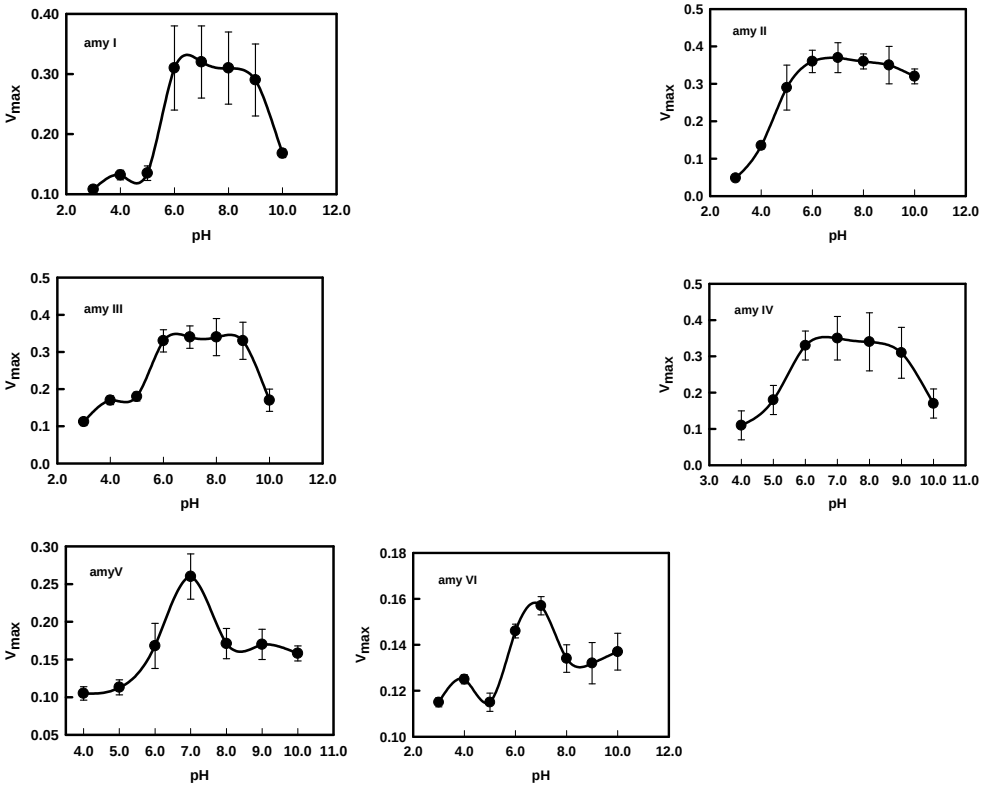
Regarding salt concentration and activity and stability, dialysis of samples in 0 M salts became in irreversible loose of activity of the enzymes. Although all of them displayed a marked halophilic character, both for activity and stability, the dependence of the kinetic parameters obtained for each salt concentration displayed quite different patterns, as shown in Figure 3. In fact, salt concentration affects V for amyI increasing its values to a plateau at 3 to 4 M NaCl, and the same with V/K, due to the little changes observed in K values (related to the affinity of the enzyme with starch). The other degrading activities suffer changes in both V and K, but leading to a final increase in V/K, related to their catalytic efficacy in degrading starch, except for amyV, the only degrading activity whose V/K decreases a little as salt concentration increased, with maximal activity at very low salt concentration. However, it was able to maintain its activity and V/K with increasing salt concentration until 3M NaCl.

Figure 3. Salt concentration (NaCl) effect on the kinetic parameters corresponding to the hydrolysis of starch catalyzed by amyI, II, III, IV, V and VI, respectively. Data was fitted to Michaelis-Menten equation to obtain V (●) (Vmax, expressed in U/mL), and V/K (■) (expressed in U/mL·(mg/mL)$^{-1}$).



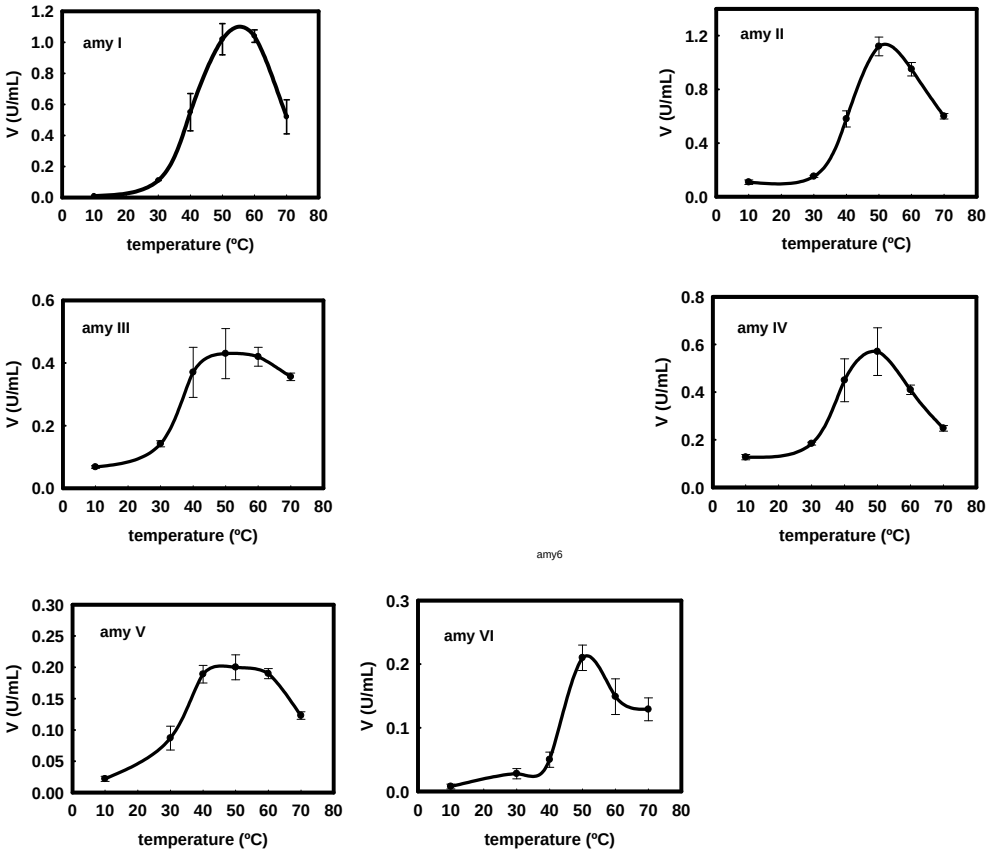
The study of activities with pH also displays differences between them. As shown in Figure 4. Profiles found in plots Vmax versus pH may be related to acid-base behavior of the residues implied in the reaction, that in the "acidic side of the profile" that should be deprotonated, and that in the basic side, protonated for an active enzyme. Except for amyII, active at pH 5, the other activities start at pH 6, and decayed at pH 9, except amyII, which activity decayed at pH 10 and both amyV and amyVI, at pH 8.

Figure 4. Effect of pH on the kinetic parameters determined at 40ºC by varying starch concentration for each pH by amyI, II, III, IV, V and VI, respectively.



Finally, their behavior with temperature, displayed in Figure 5, also presented differences in the patterns obtained: amyIII and amyV, fully active at 50ºC, and the other at 40ºC, and in the other side, amyIII still highly active at 70ºC, instead of 60ºC for the other ones. We may attribute certain thermophilic character to amyIII activity, feature that has been frequently reported for enzymes obtained from halophilic organisms

Figure 5. Effect of temperature on the kinetic parameter Vmax, determined as described in "materials and methods" at temperatures from 10ºC to 70ºC for amyI, II, III, IV, V and VI respectively.



Analysis of products

The products obtained were analyzed by different methods, mainly by thin layer chromatography (TLC). The study of these products by HPLC did not yield in all the cases clear chromatograms, which resolution was not enough to consider them significant. The other technique used was thin layer chromatography. All TLC plates developed for these products showed not a single product but a mixture of them. In Figure 6, in lane I, are displayed the products of amyI, mainly maltose, similar to amyII, that produced a mixture of maltose and glucose. The products of amyIII were mainly maltotriose, maltohexose and larger saccharides, amy IV only produced larger (counted in "glucose units")

saccharides and amyV and amyVI also produced maltohexose (or similar size). Calculation of the average sizes of the pieces produced by the enzymes, assuming the production of molecules with reducing ends from starch (non-reducing ends containing molecule) also offered an approximation of the kind of components of the final reaction mixture. Assuming homogeneity, the average size of the saccharides produced by amyI was 2.6±0.6 glucose units, similar to 2.4±0.3 for amyII. For amyIII it was 29±10 glucose units, 6.6±1.5 glucose units for amyIV, 50±10 glucose units for amyV, and 23±5 glucose units for amyVI, coherent with a mixture of saccharides of different size observable in the thin layer chromatogram in Figure VI, probably due to a random and complex degradation of the starch. The activities amy I, II y IV mainly low molecular weight oligosaccharides, meanwhile amy III, V, and VI produced larger carbohydrates; not being in contradiction with the ability of amy I and amy II, to produce glucose and maltose, meanwhile the other activities would be mainly devoted to obtain larger saccharides.

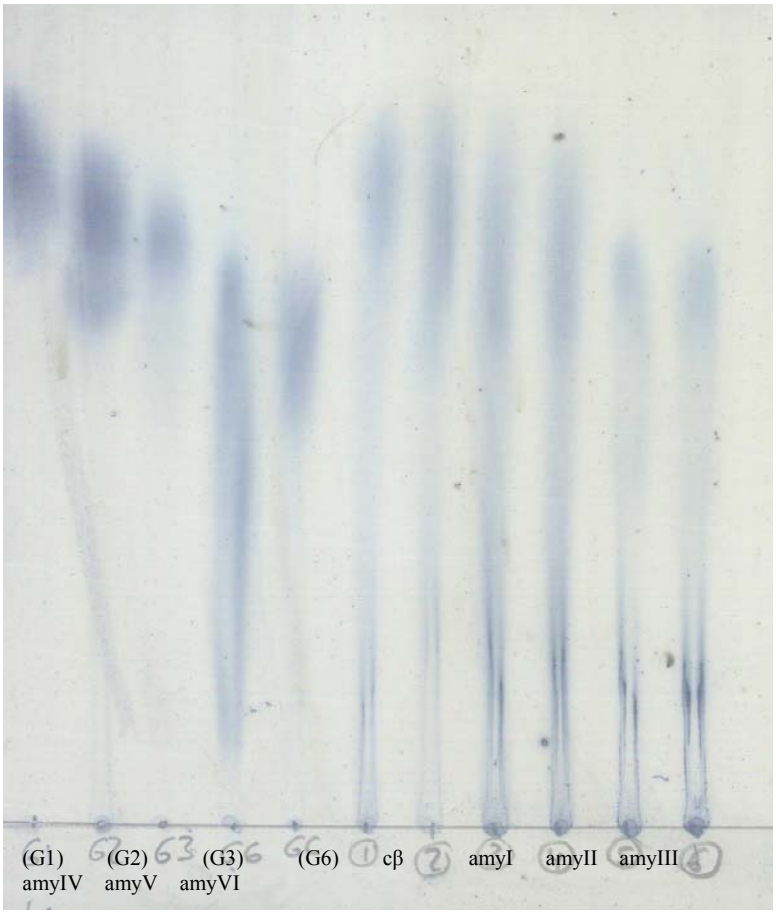


Figure 6. Thick layer chromatography plate developed with a mixture of isopropanol, ethyl acetate and water. Standards lanes: G1 glucose, G2 maltose, G3 maltotriose, G6 maltohexose, cβ ciclodextrine; sample lanes: amyI to amyVI, corresponding to the reactions with these activities, respectively.

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We attempt to assign these activities to the already found genes that encode for amylolytic activities into *Haloferax mediterranei* complete sequenced genome disposable in NCBI data bases, exposed in Table 4. These genes includes the cyclodextrin glucanotransferase, already fully characterized and described by Bautista et al. (2012)

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Table 4. Genes assigned to amylolytic activities, found into the *H. mediterranei* complete genome disposable in NCBI data bases, marked in bold the cyclodextrin glucanotransferase (Bautista et al 2012)

Gene name	Entry name	Protein name	Aa	Mr kDa	pI
amy1 HFX_0533 C439_07790	I3R200_HALMT	Alpha amylase/glucosidase	701	78.2904	4.17
Cgt	Q53I75_HALME	**Cyclodextrin glucanotransferase**	713	78.5994	4.19
amyP1HFX_0535 C439_07780	I3R202_HALMT				
amy3 HFX_1044 C439_05260	I3R202_HALMT	Alpha glucosidase	599	69.5004	4.32
amy4 HFX_1802 C439_01492	I3R5J6_HALMT	Glucan 1,4-alpha-maltohydrolase /alpha-glucosidase	698	77.207	4.28
amy5 HFX_1803 C439_01487	I3R5J7_HALMT	Glucoamylase / glycosyl hydrolase	1511	166.6422	4.56
amy2 HFX_1803 C439_01487	I3R5J7_HALMT	Glucan 1,4-alpha-glucosidase / glycosyl hydrolase	673	74.2576	4.26
HFX_1801 C439_01497	I3R5J5_HALMT	Hypotetical protein	879	98.5281	4.20

To achieve this aim, the most prominent bands in these polyacrylamide gels showed in Figure 7, we subjected them to mass-spectrophotometry studies, but none of them yielded reliable results. The peptides obtained by trypsin digestion

of these SDS-PAGE bands selected did not match significantly with already reported hydrolyzing enzymes summarized in table 4, except those from cultures in ammonium acetate medium, which matched 100% with cyclodextrin glucanotransferase sequence, referred in Table 4. This finding was consistent with the lack of cyclodextrin glucanotransferase specific activity in all the samples from cultures in potassium nitrate medium.

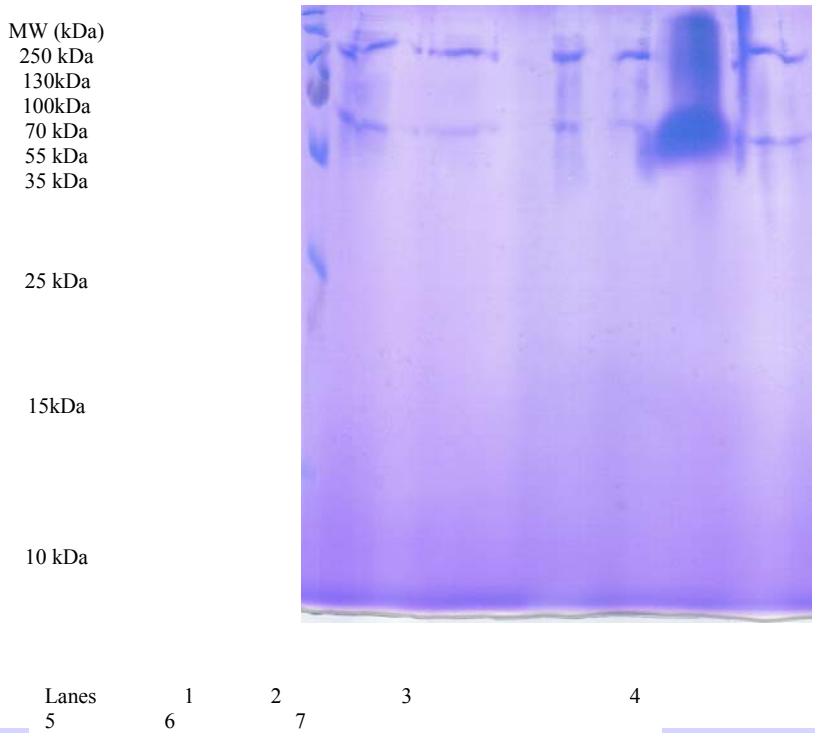

Figure 7. Patterns of bands obtained by SDS-PAGE for the different purified amylolytic activities described in Table I. Lane 1: Molecular Weight markers, Lane 2 to 7: amyI to amyVI respectively

Comment [M. Ali15]: Adjust lanes number

Comment [M. Ali16R15]:

This method obtained the peptides performing a random calculation of molecular weights accordingly with those already found in databases. May be these randomly obtained peptides are not already found in these databases as pertaining to carbohydrate hydrolyzing enzymes, and further work in still undone in peptide assignation to each gene, or even not all genes have been already found. More work would be necessary to further understand the published genomes, thus reflecting their enormous complexity, especially their proteomic aspect.

Discussion conclusions and final remarks

The purification schedule was a little different to that routinely followed for other halophilic enzymes, already discussed in previous reports (Pérez-Pomares et al. 2003, Bautista el al. 2012), but was quite similar to that used to isolate glycolytic enzymes in cell cytoplasm (Pérez-Pomares et al 2009). Moreover, starch column was chosen to select only those enzymes that interact more closely, more specifically, with starch (being able to stick to it actually), and able, on the other hand, to respond to the presence of maltose, one of the possible oligosaccharides produced in the enzymatic degradation of starch. Since the different activity peaks found in the starch column chromatograms represents the interactions between complex molecules, starch and the enzymes, as well as the interactions with maltose, each peak could not be due to different enzymes but to the different ways they may interact, corresponding to possible different conformations or points of interaction in the enzyme. Further analysis of these activities showed deep differences between them, leading to confirm the presence of different enzymes.

Although all of them behaved as typical halophilic enzymes, the effect of salt concentration on the kinetic parameters were illustrative of the variety of ways, strategies, halophilic organisms possess to affront or profit the high salinity in their natural environments. Curiously, the patterns for amy II to amy V are anyhow similar to that reported for the glutamate dehydrogenase from the extreme halophilic bacteria *Salinibacter ruber* (Bonete et al 2003), that also displayed high activity both at a relatively low and a very high salt concentration.

In addition, pH effects on these parameters were not the same for all the amylolytic activities, indicating that protonation-deprotonation of the amino acidic residues implied in the reaction occurred at different pHs and consequently there were differences in the chemical mechanism they followed. In fact, the products obtained were different for each of them: amy I, II y IV would be essential to produce glucose and maltose, and amy III, V, and VI would degrade starch more randomly, producing larger saccharides. Worthy of mention is the production of amy IV, V and VI of saccharides approaching six glucose units large, underlying the capital importance of maltohexose for our organism in order to success in its environment, as already discussed by Bautista et al (2012).

Attending to the possible role of these enzymes, as stated in the introduction, the variety of enzymes that may be related to carbohydrate degradation is enormous, making it difficult to assign the different activities found to its correspondent enzyme. To make it even more complex, available sources of carbon and nitrogen seem to determine their production in microorganisms, such as bacteria *Bacillus megaterium* (Bhutto and Dahot 2010), Bacillus

amyloliquefaciens P-001 (Deb et al. 2013), and *Brevibacillus borstelensis* R1 (Suribabu et al. 2014), fungi as Trichoderma viride BITRS-1001 (Arotupin and Ogunmolu 2012), and also archaeal microorganisms such as Rhodothermus marinus, an extreme thermophilic organism (Gomes et al 2003). The same must be applied for our extreme halophilic archaeon Haloferax mediterranei, which versatility and ability to success in variety of media has made of adequate object of several studies regarding nitrogen metabolism (Bonete et al 2007, Pire et al 2014). Its ability to grow with nitrate as unique nitrogen source enabled us to study how the organism degrades starch as solely carbon source. This feature clearly demonstrated that our halophilic archaeon poses the needed operative metabolic machinery to get fully profit of this source. Since growth in media C, i.e. ammonium, as solely nitrogen source, was not successfully achieved by this organism, the assimilation of nitrogen from nitrate must be essential to permit the organism to access to the necessary enzymes to use starch, actually its only available carbon source.

This adaptation implied the production of different battery of enzymes, and also excreted at variable general amylolytic activity in the external media. Both, the number of activities, and the total starch degrading activity was higher in nitrate containing medium than in ammonium one. Since the organism is also known to grow optimally in ammonium sulphate and glucose controlled media (Pire et al 2014), as well as in ammonium acetate containing medium (Medium C), we may conclude that *Haloferax mediterranei* is an organism that is not able to use starch optimally when ammonium is the solely nitrogen source, and the use of starch and nitrogen source is not independent each other. This ability was probed vital for the organism to grow in each medium.

Moreover, different activities with putative amylolytic activity are reported in the published genome of this halophilic archaeon, *Haloferax mediterranei*, assigned nearly all of them by computational comparative studies as putative amylolytic genes, corresponding to activities which role has to be related to the abilities to degrade, or modify to be degraded, carbohydrates such as starch. In fact, only one out of these six sequences, the glucotransferase, has been assigned to the external activity characterized by Bautista et al. (2012). The others remain uncharacterized. Amylolytic activities excreted strongly depend on, not only carbon sources, but also on the nitrogen compounds available in the media.

Nitrogen sources nitrate and ammonium strongly determine the metabolic pathways working for each set of conditions and influence the yield of production of amylolytic enzymes. Ammonium nitrate and sodium nitrate are the best nitrogen sources for maximum amylase production in microbial enzymes (Mahmood & Rahman 2008; Deb et al. 2013). Besides the different

amount of enzyme excreted to the medium, the different set of enzymes implied in each case may differ depending on this nitrogen source. We need their isolation and characterization to better understanding the way microorganisms, in this case extreme halophilic archaea one, deals with the conditions they have to endure and succeed.

In conclusion, composition of media strongly determines the set of enzymes employed by the organism to profit starch. Learning more about how our organism get optimal profit of starch, implies knowing more about the great variety of amylolitic enzymes present in their genome.

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