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

ABSTRACT

The use of starch as well as other carbohydrates is essential for the cell in order to obtain carbon and energy, mainly when it is exposed to extreme medium conditions. The halophilic archaeon Hfx mediterranei is a good example of optimal adaptation to these limiting media, since it has been reported to use many different carbon and nitrogen sources.

The adaptation of the halophilic archaeon to these media: starch, glycerol and yeast extract maximal medium by producing glycosidic activities that may be intra and extracellular has been extensively studied and compared.

The purification steps for many halophilic enzymes are those developed for decades by Bonete et al. including ion exchange, gel affinity gel filtration chromatographies and elution of the sample loaded in a Sepharose 4B column, by changing hydrophobic interaction.

Once isolated, their kinetics at different pHs, substrates, temperatures and regulatory metabolites were determined, confirming in nearly all the cases a typical halophilic behavior, requiring high salt concentration for optimal activity and stability in a range 1.5 to 3 M NaCl., as well as certain thermophilic character, with optimal activity at 50ºC to 60ºC.

At least six different activities, with molecular masses in a range of 15 kDa to 80 kDa, were excreted in the nitrate-containing medium, but not all of them found in the ammonium containing one. These enzymes displayed a different affinity for starch as a chromatographic matrix, when eluted with maltose gradient, and differed in their kinetic parameters for starch as a substrate. In addition, three different amylolitic activities were detected in the cytoplasm of the organism grown in a starch containing medium.

These findings show a complex pattern of use of different enzymes to achieve a maximal profit of the sources available, also adapted to the specific media the organism has to endure, mainly the nitrogen source present in the medium. However, the assignation of each enzyme to the possible sequences in the genome only could be achieved for the cyclodextrin glycosyltransferase. For the rest of them, it still unsolved.

*Keywords: Halophilic archaea • Haloferax mediterranei • Amylolytic enzymes • Isolation and biochemical characterisation • carbon and nitrogen sources.*

**1. 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 the available glucose and a wide variety of oligosaccharides obtained from carbohydrate macromolecules (Vihinen and P. Mäntsälä 1989, Bonete et 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 hydrolysed into smaller oligosaccharides by other glycosylases. The enzymes responsible for these transformations belong, mostly to the 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 cleaves 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 an 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 an isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41), only hydrolyse 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,namely, α−, β- and γ-cyclodextrins (Bonete et al 2007).

Classifying this great variety and number of carbohydrate-active enzymes into their sequence-based families, and based on the obtained knowledge (as catalogued by the CAZyme database; http://www.cazy.org/), Stefan Janeˇcek (2023) considered that α-amylase enzyme specificity is present in four CAZy glyoside hydrolase (GH) families: GH13, GH57, GH119, and GH126. Particularly, the two former α-amylase families, GH13 and GH57, cover, in addition to α-amylase, most of the numerous amylolytic enzymes, such as α-glucosidase, pullulanase, amylopullulanase, isoamylase, cyclodextrin glucanotransferase, 4-α-glucanotransferase, α-glucan branching enzyme, α-glucan debranching enzyme, trehalose synthase, sucrose isomerase, etc., while two other “amylases”, β-amylase and glucoamylase, constitute their own families, GH14 and GH15, respectively (Stefan Janeˇcek 2023).

Due to the extensive research over the last years, insight is emerging into the sugar metabolism of Archaea in general, but that of hyperthermophiles in particular. Most of hyperthermophilic Archaea are heterotrophs that use polypeptides as carbon and energy sources. However, a lot of archaeal species are also able to grow efficiently on different polysaccharides, including starch, as well as on oligosaccharides and monosaccharides (Verhees el al. 2003). So, starch and other saccharides degrading enzymes, such as amylases, became essential for this ability, playing a central role in carbohydrate metabolism.

Our report is focused on the starch and other carbohydrate degrading enzymes from the halophilic archaeon Haloferax mediterranei. This extremophile has been reported to grow efficiently on starch, lactose, sucrose, fructose and glucose (Danson 1989; Rodríguez-Valera et al. 1983; Altekar and Rangaswamy 1990) and has previously been reported to grow in minimal medium with starch as carbon and energy source (Pérez-Pomares et al. 2003). Although a lot of effort has been invested in these degrading enzymes from hyperthermophilic archaeal organisms, fewer studies are focused on their counterparts from other physiological groups of extremopliles such as halophiles and alkaliphiles (Mijts and Patel 2002). The enzymes from halophilic organisms are adapted to high salt concentration and use to be active and stable at relatively high temperatures above 60ºC. Due to these features, these enzymes have an enormous industrial potential, yielding suitable enzymes for catalyzing starch and other carbohydrates hydrolysis under unusual conditions such as high ionic strength and temperature. There is a great variety of enzymes implied in the hydrolysis of starch and are widely used in industrial processes and in biotechnology, especially in the food and starch processing industry (Vihinen and Mäntsälä 1989; Kadziola et al. 1998; Machius et al. 1995).

Our halophilic archaeon can produce several extracellular starch degrading enzymes when it was grown in a variety of controlled medium. The first degrading enzymes characterized were: an α-amylase (Pérez-Pomares et al. 2003), and a cyclodextrin glycosyltransferase (Bautista et al. 2012). This last activity was the further studied and the only which sequence was assigned by Bautista et al (2012). This glycosyltransferase may act degrading and transforming starch in several ways, including both coupling and disproportionation activities, but cyclisation 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 members 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 produced a mixture of different dextrins and maltose. Its behaviour is very similar to the amylases from Halobacterium salinarum (Good and Hartman 1970), Natronococcus amylolyticus (Kobayashi et al. 1992), the halophilic bacterium Bacillus Siamensis Sp. F2 (Rathod et al 2024), and to that from the moderately halophilic bacteria Halomonas meridiana (Coronado el al 2000).

Besides, grown in a starch-containing medium, it 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 a media containing ammonium acetate and starch, except AMY2, detected when the organism was in a medium with glycerol as a carbon source (Pérez-Pomares et al 2009). The degradation of starch in an 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 succeeds in getting a profit of the starch, in different conditions of growth, with different nitrogen sources. Regretfully, the other activities have not been already assigned.

**2. material and methods**

**2.1 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 temperature and a pH of 7.2 and supplemented with different carbon and nitrogen sources. Three different media were 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 contained 1% ammonium chloride and starch 0.2% (w/v); and medium C contained 1% (w/v) ammonium acetate, source of both carbon and nitrogen, and supplemented with 0.2% (w/v) soluble starch (adding a previously filter-sterilised starch stock solution). The clarified medium 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.

**2.2 Activity determination**

**2.2.1Determination 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). The adequate starch concentration in the reaction mixture was adjusted with potato-soluble starch (Sigma) and terminated it by cooling in ice. Colour 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 hydrolysed 1 mg of starch in 1 min (Haseltine et al. 1996).

The activity was also measured 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. The development of color was measured spectrophotometrically at 540 nm. One unit of activity was defined as the amount of protein, which produced 1 µmol of reducing ends in 1 min. Maltose was used for a standard curve. All assays were performed, at least, in duplicate and average values obtained.

**2.2.2 Cyclodextrin glycosyltransferase activity assay**

The cyclisation activity was determined using different dyes: methyl orange, phenolphthalein and bromocresol green. The production of cyclodextrins was analysed spectrophotometrically by the absorbance decrease 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 cyclisation activity (U) is defined as the amount of enzyme that produces 1 µmol of a-, b- or c-CD, as described by Bautista et al. (2012).

**2.3 Enzyme isolation and purification**

Several methods were applied to concentrate the excreted enzymes or to isolate them from the initial enzyme sample. The election of each particular method also determined which enzyme or enzymes composed the obtained sample.

**2.3.1 Isolation and purification of excreted enzymes**

The external medium once clarified by centrifugation and discarded the pellet, was used as initial sample, and the enzymes were isolated and concentrated using two different initial methods:

*- Hydroxyapatite column.* The supernatant from 2 l of culture was treated with hydroxylapatite previously equilibrated with 50 mM Tris-HCl buffer pH 7.0, 3 M NaCl. The proteins bound to hydroxylapatite were recoveredby centrifugation at 3,000 rpm for 5 min. The enzyme was eluted from hydroxylapatite in 10 ml of 0.2 M phosphate buffer, pH 7.5, and 2.5 M ammonium sulfate and was used as starting material for next purification steps: Sepharose-4B column, eluted using a linear gradient from 2.5 M ammonium sulfate to 0.5 M ammonium sulfate containing 20% (w/v) glycerol. The more active fractions were pooled and concentrated using a DEAE-cellulose column and gel filtration in a Sephadex G-50 column, as described by Pérez Pomares et al. (2003). This was the first method used, and allowed to purify and to characterize an α-amylase ( Pérez-Pomares et al 2003)

- β-CD-Sepharose 6B chromatography. The supernatant was concentrated by tangential ultrafiltration, using VivaFlow 200 filtration system with a 30 kDa cut-off membrane (Vivascience), followed by β-CD-Sepharose 6B chromatography. Protein elution was carried out with an increasing linear gradient of 0–3 mg/ml β-CD in buffer A at a flow rate of 30 ml/h, and finally Sephacryl S-200 chromatography, as described by Bautista el al. (2012). This step allowed the purification, characterization and genomic assignation of the corresponding Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) (Bautista el al 2012)

- *Starch column*. The supernatant from 100 mL culture was passed through a starch column prepared with insoluble starch packed in a 2.5 x 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 washoff 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. A discontinuous gradient of crescent concentrations of maltose was used for better isolation of the different enzymes and used for further studies. Amylase activity was tested in all the fractions and pooled the more active, Sephacryl S-300 and a Sepharose 4-B gel filtration column that also served to determine its molecular weight. Protein concentration was determined 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 analysed by SDS-PAGE for purity and subunit molecular weight.

**2.4 Analysis of reaction products**

Thin-layer chromatography (TLC) was used in order to analyse the products of the different amylolytic activities. Each purified enzyme was incubated overnight in the reaction mixture, as described previously, and spotted in silica gel plates. Each plate was developed with solvent mixtures containing different proportions of isopropanol–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, dissolved 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 analysed by gel filtration in a 2.5 × 30 cm Sephadex G-100 column. The lower molecular weight products were further analysed 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 consisted of maltose, maltotrioside, and maltohexaoxide prepared in concentrations of 1 mM in the same conditions as the reaction products.

The α-amylase stereo-specificity was determined by NMR spectroscopy Spectra were recorded on an Avance DRX-300 nuclear magnetic resonance (NMR) spectrometer (Bruker, Germany). Optical rotation (specific rotatorial activity, [α]D20) was registrated in a digital polarimeter (Jasco DIP-1000) as described by Pérez-Pomares et al (2003)

**2.5 Effect of salt concentration, pH, and temperature**

The enzyme activity was tested 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).

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 the study of pH, different buffers were used: 0.2 M citric acid/ phosphate for pH ranging from 4.5 to 7, 0.2 M Tris-HCl buffers for pH ranging from 7 to 9, and 0.2 M CHES buffers for pH ranging from 9 to 10. Each of them contained 3 M NaCl. The pH was checked after each reaction but 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 temperature, starch concentrations varied from 0.5 to 5 mg/mL.

**2.6 Inhibition by maltose**

The inhibition by maltose was tested 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 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.

**2.7 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 Eq. 1 to obtain values for 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).

(equation 1)

Data for the pH dependence of V and V/K were fitted to Eq. 2 using the algorithm of Marquardt-Levenberg with the SigmaPlot program (Jandel Scientific, v. 1.02):

(equation 2)

where Y represents the value of V or V/K for each pH and C is the pH-independent value of the parameter at the optimum state of protonation. K1 and K2 are dissociation constants associated with ionizing groups that, to show activity, must be protonated or deprotonated, respectively.

The values of pK obtained at different temperatures were fitted to Eq. 3:

(equation 3)

in order to determine values for the ionization enthalpy (ΔHºion )

and the ionization entropy ΔSº. Data from the stability studies, salt concentration, pH, and temperature, 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 denaturating process were determined from the slope of the straight lines obtained

Same processing was followed with data for each maltose concentration. In the study of the inhibition of the enzyme with maltose, the inhibition constant was determined by adjusting the data to the competitive inhibition reaction.

(equation 4)

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.

**2.8 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 analysed using the BLAST database (http://www.ncbi.nih.gov/BLAST)cond level heading.

**3. results and discussion**

**3.1 Amylolytic activities excreted into different media.**

For each medium, containing nitrate A, ammonium chloride B, and ammonium acetate C, different total starch degrading activity was obtained. This can be due to the amount and the concentration of the enzymes excreted or also due 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 grown in medium A was 2.5 ± 0.5 g wet cells per 100mL culture, similar to that found in medium C, 2.2 ± 0.5 g. This was much higher than that found for medium B, i.e., 0.5 ± 0.15 g per 100mL culture. However, in spite of this residual growth, amylolytic activities were detected in it. The posterior purification steps followed, described in materials and methods section leaded to an isolation of a different set of degrading activities. This may be to an amazing variety of processes of the degradation, uptake and metabolism of starch, possibly due to the continuous production of saccharides and oligosaccharides of different chain lengths.

**3.2 Isolation of an α-amylase using an hydroxyl-apatite column .**

When the supernatant of a culture in medium C was treated with hydroxyl-apatite, previously equilibrated with 50 mM Tris-HCl buffer pH 7.0M NaCl, the containing ammonium acetate as main degrading activity found in this medium, nitrogen source, was an halophilic α-amylase, characterized by Pérez-Pomares et al (2003). The steps followed included the recovery of the proteins bound to hydroxyl-apatite by centrifugation at 3,000 rpm for 5 min and eluted from hydroxyl-apatite in 10 ml of 0.2 M phosphate buffer, pH 7.5,and 2.5 M ammonium sulphate. The active pooled fractions were applied to a Sepharose-4B column and eluted using a linear gradient from 2.5 M ammonium sulfate to 0.5 M ammonium sulfate containing 20% (w/v) glycerol. Finally, the enzyme sample obtained was further purified by gel filtration in a Sephadex G-50 column. The optimal yield was obtained when the cells were grown in a minimal medium containing ammonium acetate at concentrations from0.5 to 1.0% (w/v) plus soluble starch. Only one main activity was obtained, that was further characterized. The HPLC analysis of the products of the reaction (during 1 h) showed that the main products were maltose (65%), in lower proportion maltohexaose (9%), and other minority components. The NMR spectra were recorded for both the reaction mixture without enzyme and the reaction mixture after the reaction with a-amylase in deuterated 10 mM phosphate buffer, pH 7.3. In the anomeric proton region of p-nitrophenyl a-D -maltohexaoside (Fig. 1) appeared a proton signal of the hydrolyzed product at 5.2 ppm (doublet, J=3.57 Hz) that did not appear in the control spectrum. Optical rotation of the reaction solution containing initially 1% soluble starch decreased sharply after the complexion of the reaction from a specific rotatorial activity [α]D20 +141.5 to [α]D20 +56.5. The purification of the enzyme was analyzed by SDS-PAGE, and one main band consistent with the value obtained by gel filtration was observed. The relative molecular mass for this band was 58±2 kDa. This method usually yields abnormally high molecular weight values for halophilic enzymes, so this was an overestimated value for this parameter The relative molecular mass of the enzyme, determined by gel filtration, is 50±4 kDa, indicating that the halophilic α-amylase is a monomeric enzyme. The enzyme showed a salt requirement for both stability and activity, being stable from 2 to 4 M NaCl, with maximal activity at 3 M NaCl. The enzyme displayed maximal activity at pHs from 7 to 8, and its optimal temperature was in a range from 50 °C to 60 °C. The results also implicated several prototropic groups in the catalytic reaction. The pK 1 values for the log V profiles, as well as those for log V/K, at different temperatures allowed us to determine the ionization enthalpy of the groups involved in the α-amylase reaction. The pK 1 values, related to a group that must be deprotonated for binding or catalysis, were in the same range as those found in other proteins of an imidazolium group (5.5–7.0). The enthalpy of ionization calculated was also very close to the values that this prototropic group, in histidine residues, displays in other enzymes (6.9–7.5) (Segel 1993). The pK 2 values for the log V and log V/K also decrease as the temperature increases. However, the ionization enthalpy for both parameters from the dependence of pK 2 on temperature was far different. These pKs for the basic side of the pH proﬁles, related to a group or groups preferentially protonated for catalysis, are in the same range as those found for the phenolic OH of a tyrosine (9.8–10.5) or the ϵ-amine of a lysine (9.5–10.6). The ionization enthalpy for the phenolic group is about 6 kcal/mol, very close to that obtained for the log V proﬁle. The pK 2 from the log V/K proﬁle was very similar to the ionization enthalpy for the ϵ-amine, which ranged from 10 to 13 kcal/mol (Segel 1993). The optimal pH range for the catalytic reaction ranged from 7 to 8. These values are compared with those reported for other α-amylases (Pérez-Pomares et al 2003)

**3.3 Isolation of a cyclodextrin glycosyltransferase using a β-CD-Sepharose 6B column .**

H. mediterranei was grown to stationary phase in medium C, containing ammonium acetate as nitrogen source, and soluble potato starch, and pH 7.2. Cells were harvested by centrifugation and supernatant was used as crude enzyme preparation for the following steps: Step 1: Tangential ultrafiltration. Supernatant was concentrated using a 30 kDa cut-off membrane (Vivascience). Next step, Step 2 was a β-CD-Sepharose 6B chromatography, prepared by coupling β-CD (Sigma) to epoxy-activated Sepharose 6B (GE Healthcare). The elution was carried out with an increasing linear gradient of 0–3 mg/ml β-CD. The final step, Step 3 was S-200 gel filtration chromatography, which also was used to determine the molecular mass of the enzyme. The enzyme found in these conditions was A cyclodextrin glycosyltransferase (CGTase,EC 2.4.1.19) was successfully isolated and characterized from the halophilic archaeon Haloferax mediterranei. The enzyme is a monomer with a molecular mass of 77 kDa andoptimum activity at 55°C, pH 7.5 and 1.5 M NaCl. The enzyme displayed many activities related to the degradation and transformation of starch. Cyclization was found to be the predominant activity, yielding a mixture of cyclodextrins, mainly a-CD, followed by hydrolysis and to a lesser extent coupling and disproportionation activities. Gene encoding H. mediterranei CGTase was cloned and heterologously overexpressed. Sequence analysis revealed an open reading frame of 2142 bp that encodes a protein of 713 amino acids. The amino acid sequence displayed high homology with those belonging to the a-amylase family. The CGTase is secreted to the extracellular medium by the Tat pathway.

Upstream of the CGTase gene, four maltose ABC transporter genes were also sequenced (malE, malF, malG, malK) by Bautista el al (2012). The expression of the CGTase gene yielded a fully active CGTase with similar kinetic behaviour to the wild-type enzyme. The H. mediterranei CGTase was the first halophilic archaeal CGTase characterized, sequenced and expressed (Bautista el al 2012)

**3.4 amylolitic activities detected in the cytoplasm of the organism.**

Three different amylolitic activities, designated AMY1, AMY2 and AMY3 were detected in the cytoplasm of the extreme halophilic archaeon Hfx. mediterranei grown in a starch containing medium. This organism had also been reported to excrete into the external medium an α-amylase in such conditions. The presence of these different enzymes which are also able to degrade starch may be related the use of the available carbohydrates and maltodextrins, including the products obtained by the action of the extracellular amylase on starch that may be transported to the cytoplasm of the organism. Two of these glycosidic activities were also purified and further characterized. As other halophilic enzymes, they were salt dependent and displayed maximal activity at 3M NaCl, and temperature 50ºC. The crude enzyme preparation was applied to a Sepharose-4B column and eluted using a linear gradient from 2.5 M ammonium sulfate to 0.5 M ammonium sulfate containing 20 % (w/v) glycerol. The more active fractions were pooled and further purified. Each pool was applied to a starch column prepared with insoluble starch packed in a 2.5x10 cm column. This column was intensively washed and the enzyme recovered with 0.5M maltose prepared in the same buffer. The active fractions pooled were further purified by gel filtration in a Sephacryl S300 column.

The degrading enzyme marked as AMY2 did not by significantly bind to the starch column. In this case, the sample was concentrated with a DEAE-cellulose column and the enzyme further purified by gel filtration on Sephacryl S300 column. The active fractions were concentrated to a final volume of 50 microliters by ultrafiltration in an Amicon concentrating unit using a 10,000-MW cut-off membrane, and the enzyme applied to a SDS-PAGE for further studies. The activity AmyII was also detected in extracts from cells grown in media with glycerol instead of starch and in rich medium. This enzyme was able to degrade starch yielding small oligosaccharides and displayed similar halophilic behavior with salt requirement in a range 1.5 to 3 M NaCl.

Their molecular weights, estimated by gel filtration on a Sephacryl S300 column, and kinetic parameters for starch and maltohexose are those displayed in table 3.4.1.

Table 3.4.1. Estimated molecular weights and kinetic parameter for starch and maltohexose for the degrading activities present in the cytoplasm of *H. mediterranei*.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | MW (kDa) | Km starch (mg/ml) | V/K starch | Km maltohexose (mM) | V/K maltohexose |
| AMY1 | 38 ± 3 | 16 ± 4 | 3.7·10-4 ± 0,2·10-4 | 2.2 ± 0.6 | 1.3·10-3 ± 0.2·10-3 |
| AMY2 | 116 ± 34 | 11 ± 2 | 2.8·10-4 ± 0.2·10-4 | 0.35 ± 0.08 | 7.1·10-3 ± 1.3·10-3 |
| AMY3 | 64 ± 7 | 1.1 ± 0.3 | 2.7·10-4 ± 0.4·10-4 | 13 ± 5 | 2.5·10-3 ± 0.7·10-3 |

Thin layer chromatography (TLC) plates were developed at different mixtures of isopropanol, acetone and lactic acid. When mixed in 4:2:1 proportion respectively, the separation of oligosaccharides of lower molecular weight, such as glucose (G1) and maltose (G2) was optimal. To get an optimal separation of oligosaccharides from G1 to G6 (maltohexaose) the mixture used was 2:2:1 (v/v) isopropanol, acetone and lactic acid respectively. The degradation of starch by AMY1 yielded maltose in a very low concentration and dextrins larger than maltohexaose because they could not be separated by TLC in the conditions tested. The degradation of maltohexaose by AMY1 yielded so low concentration of reducing ends that were no detectable by TLC. The degradation of starch by AMY2 produced mainly glucose and dextrins larger than maltohexaose, the degradation of maltohexaose produced also glucose. The enzyme AMY3 degraded starch producing mainly maltotetraose or maltopentaose and dextrins larger than maltohexaose and produced no detectable amounts of oligosaccharides from its reaction with maltohexaose to be detected under the conditions the TLC was performed. The role of these intracellular carbohydrate degrading enzymes, that display amylolitic activity, may be related to the degradation of dextrins or other carbohydrates available in the inside of the cell, depending on the substrates they have to degrade or the products they have to provide the organism.

**3.5 Isolation of starch degrading activity was obtained using a starch column .**

For each medium, containing nitrate A, ammonium chloride B, and ammonium acetate C, different total starch degrading activity was obtained. This can be due to the amount and the concentration of the enzymes excreted or also due 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 grown in medium A was 2.5 ± 0.5 g wet cells per 100mL culture, similar to that found in medium C, 2.2 ± 0.5 g. This was much higher than that found for medium B, i.e., 0.5 ± 0.15 g per 100mL culture. However, inspite of this residual growth, amylolytic activities were detected 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, (Fig. 1A).

Figure 1A. Chromatography of the 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 a nitrogen source. For all the fractions collected, the volume was 3 mL.



A



B

Medium C was used exclusively as growth and amylolytic activity control, since previous studies had already been reported, including the full characterisation of two external amylolytic enzymes, viz., α-amylase (Pérez-Pomares et al 2003) and cyclodextrin glycosyltransferase (Bautista el al 2012). Moreover, medium C contained nothing but two different carbon sources (acetate and starch). In order to study the exclusive use of starch as a carbon source, further studies were focused on the starch degrading enzymes excreted in media A and B, where the set of enzymes permitted full usage of starch as an exclusive carbon source.

The results in Fig. 1B again display 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 the number of the enzymes implied led us to pay attention only to the more prominent activities. The growth of the organism in ammonium chloride produced general enzymes that eluted at higher maltose concentration, producing practically undetected activity at a concentration higher than 0.2M maltose. The main activities were amyIII amy IV and AmyV. However, no significant cycling activity (cyclodextrin glycosyltransferase activity) was found in this ammonium containing medium (B), contrary to what was found in the acetate of ammonium containing medium (C).

The chromatography produced partially purified samples (Table 1), with a high purification factor, and further purified as previously described. The last purification step also served to estimate their molecular weight.

**3.5.1 Isolation and further purification of amylolytic activities excreted .**

The chromatography produced partially purified samples (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 3.5.1. Purification of the excreted external amylolytic activities 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 column | 21 | 0.42 | 4,9 | 41·10-3 | 10.2 | 1.4 |
|  | Sephacryl-S300 | 14 | 0,035 | 39 | 1.0·10-3 | 35 | 4,9 |
| amyII | starch column | 18 | 1.10 | 11 | 29·10-3 | 37.9 | 5.3 |
|  | Sephacryl-S300 | 6 | 0.086 | 28 | 2.0·10-3 | 43 | 6.0 |
| amyIII | starch collumn | 9 | 0.80 | 4 | 19·10-3 | 42.1 | 5.8 |
|  | Sephacryl-S300 | 16 | 0.11 | 73 | 1.8·10-3 | 61.1 | 8.5 |
| amyIV | starch collumn | 18 | 0.99 | 9.9 | 16·10-3 | 61.9 | 8.6 |
|  | Sephacryl-S300 | 12 | 0.12 | 48 | 0,83·10-3 | 144.5 | 20 |
| amyV | starch collumn | 15 | 0.84 | 7 | 61·10-3 | 13.8 | 1.9 |
|  | Sephacryl-S300 | 6 | 0.114 | 27 | 5.0·10-3 | 22.8 | 3.2 |
| amyVI | starch collumn | 15 | 0.78 | 4,9 | 0.09 | 8.6 | 1.2 |
|  | Sephacryl-S300 | 10 | 0.07 | 30 | 7.2·10-3 | 9.7 | 1.4 |

The obtained molecular masses summarised in Table 3.5.2, depended on the matrix gel, dextrose gels Sephacryl S300 and Sepharose 4B, used, and so They are considered 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 (Fig. 7). Attending to these values (Table 3.5.2), and position of the main bands (Fig. 7), corresponding to the amylolytic enzymes, all of them should be considered as monomeric enzymes.

Table 3.5.2. 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 |

**3.1.2 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 were similar to amyII as shown in Fig. 2 .



Figure 2. 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

Table 3 summarises the kinetic parameters from the equation for competitive inhibition. The Michaelis-Menten parameters, calculated by fitting the 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 behaviour 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, maltose interactions with the starch and/or enzymes, may not be necessarily related to the catalytic core of these enzymes. Moreover, the different behaviour of these activities regarding inhibition by maltose was indicative of concurrence of several enzymes implicated in starch degradation and processing.

Table 3. Kinetic parameters and the inhibition constant Ki for maltose as a 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 |

**3.1.3 Salt, pH and temperature requirements.**

Regarding salt concentration and activity and stability, dialysis of samples in 0 M salts resulted 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 (Fig. 3). In fact, salt concentration affected 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 suffered changes in both V and K, but led 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 decreased 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 were 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 displayed differences between them. As shown in Fig. 4 profiles found in plots Vmax versus pH may be related to the acid-base behaviour 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, activated at pH 5, the other activities started at pH 6, and decayed at pH 9, except amyII, which decayed at pH 10. Both amyV and amyVI , decayed 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.



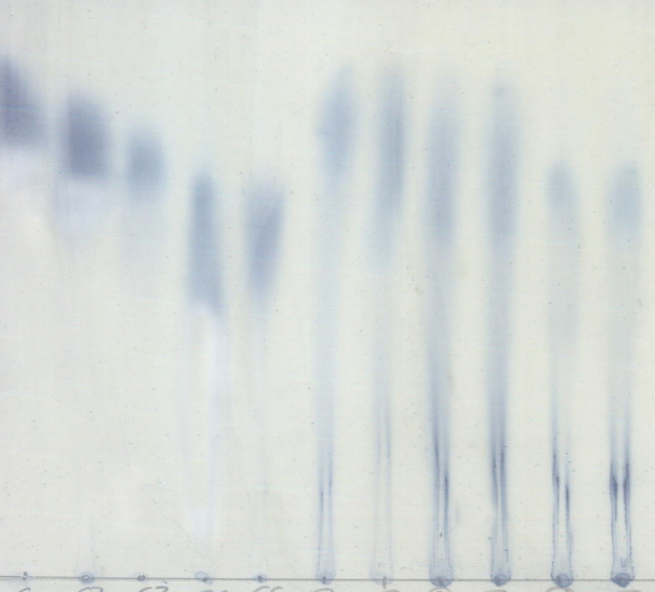
Finally, their behaviour with temperature have been displayed in Fig. 5. The results presented differences in the patterns obtained: amyIII and amyV were fully active at 50ºC, and the other at 40ºC, and in the other side, amyIII still highly active at 70ºC, instead of 60ºC for the other ones. It may be attributed certain thermophilic character to amyIII activity, a 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 analysed 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 Fig. 6, lane I, the products of amyI, mainly maltose, similar to amyII, are displayed, which 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, amyIV, amyV and amyVI, the values were 29±10 glucose units, 6.6±1.5 glucose units , 50±10 glucose units and 23±5 glucose units, respectively, coherent with a mixture of saccharides of different size observable in the thin layer chromatogram (Fig. 6), probably due to a random and complex degradation of the starch. The activities of amy I, II, IV produced mainly low molecular weight oligosaccharides; meanwhile amy III, V, and VI produced larger carbohydrates. This was not in contradiction with the ability of amy I and amy II, to produce glucose and maltose, while the other activities would mainly be devoted to obtain larger saccharides.



**G1 G2 G3 G6 cβ amyI amyII amyIII amyIV amyV myVI**

**● ● ● ● ● ● ● ● ● ● ●**

**Figure 6.** Thin 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.

An attempt was made to assign these activities to the already found genes that encode for amylolytic activities into *Haloferax mediterranei* complete sequenced genome disposable in NCBI databases. This has been exposed in Table 4. These genes include the cyclodextrin glucanotransferase, already fully characterised and described by Bautista et al. (2012)

Table 4. Genes assigned to amylolytic activities, found into the *H. mediterranei* complete genome disposable in NCBI databases, 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 were subjected to mass-spectrophotometry studies, but none of them yielded reliable results (Fig. 7). The peptides obtained by trypsin digestion of these selected SDS-PAGE bands, did not match significantly with already reported hydrolysing enzymes , except those from cultures in ammonium acetate medium, which matched 100% with cyclodextrin glucanotransferase sequence, (Table 4). This finding was in consistent with the lack of cyclodextrin glucanotransferase specific activity in all the samples from cultures in potassium nitrate medium.

**MW (kDa)**

**250**

**130**

**100**

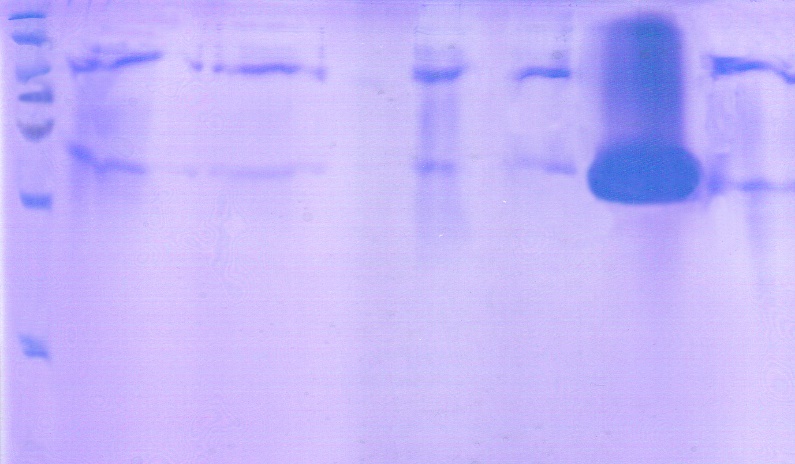
**70**

**55**

**35**

**15**

**Lanes 1 2 3 4 5 6 7**



**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

This method obtained the peptides performing a random calculation of molecular weights accordingly with those already found in databases. These randomly obtained peptides may not be found in these databases as pertaining to carbohydrate hydrolyzing enzymes, and further work is 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.

**4. Conclusion**

It is remarkable that the purification methods followed led to the recovery of different enzymes. Starch is a macromolecule able to strongly interact with a wide variety of biological molecules. This characteristic may use it in a more general, or less specific, affinity chromatography. This method, based on a specific binding interaction between an immobilized ligand and its binding partner, was also performed, using a β-CD-Sepharose 6B column, to specifically isolate a cyclodextrin glycosyltransferase (Bautista el al. 2012). Of course, the schedule also included other steps routinely followed for other halophilic enzymes. And was quite similar to that used to isolate glycolytic enzymes in cell cytoplasm (Pérez-Pomares et al 2009). Curiously, the purification following a general halophiic protocol led to the isolation of only an α-amylase (Pérez-Pomares et al. 2003),

Moreover, the starch column was chosen to select only those enzymes that interact more closely and specifically, with starch (being able to stick to it actually), and on the other hand, are able 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 represented the interactions between complex molecules, starch and the enzymes, as well as the interactions with maltose, each peak observed not due to different enzymes but due to the different ways of their interaction, corresponding to the 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 was 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 were 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, the effects of pH on these parameters were not same for all the amylolytic activities, indicating that protonation-deprotonation of the amino acidic residues implied in the reaction occurred at different pH 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. IV would be essential to produce glucose and maltose, and amy III, V, and VI would degrade starch more randomly, producing larger saccharides. This is worthy to mention that the production of amy IV, V and VI of saccharides approaching six large glucose units, proves the high importance of maltohexose and β-ciclodextrine for our organism to succeed in its environment, as already discussed by Bautista et al (2012). Due to the use of cyclodextrinegluconotransferases (CGTases) in industry manufacturing processes, its production by different organisms and conditions has been extensively studied. It has been reported that its formation depends on the presence of starch, but, curiously, inhibited by glucose (Dias-Mendel Allode et al 2024). It would explain the importance of acetate, instead of glucose, for our halophilic organism, to get optimal production of this enzyme in medium C , and not to found this activity in nitrate containing medium B.

On the other hand, for the medium B, containing nitrate as nitrogen source, and attending to the possible role of these enzymes, and 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 the extreme halophilic archaeon Haloferax mediterranei, whose versatility and ability to succeed in a variety of media have made of the adequate object of several studies regarding nitrogen metabolism (Bonete et al 2007, Pire et al 2014). Its ability to grow with nitrate as a unique nitrogen source enabled us to study how the organism degrades starch as a solely carbon source. This feature clearly demonstrated that the halophilic archaeon possess the needed operative metabolic machinery to get full profit of this source. Since growth in medium B, i.e., ammonium chloride, as a sole 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, its only available carbon source. However, when acetate itself is available, medium C, the growth is optimal as well as the production and excretion of amylolytic enzymes to the external medium. This enzymes, α-amylase and have been already described in this chapter. This findings are consistent with the results obtained for amylolytic enzymes found in medium B, amyIII, amyIV and amyV are the main enzymes found, and the products of these activities includes maltohexose or β ciclodextrine, althoug these metabolites are not enough to enable a full growh of the cell culture in medium B. The use of acetate, in this case, seems to be essential to enable the profit of bot, amonnium and starch, as could be seen in cell grown in medium C, corroboring the interconexion between carbon and nitrogen sources.

Since the organism is also known to grow optimally in ammonium sulphate and glucose controlled media (Pire et al 2014), it seems that the organism has any problems to get glucose in enough amount from starch, consistent with the less activity found of the enzymes amyI and amyII in medium B. Again it can be concluded that the nitrogen source and carbon sources are not independent of each other.

Adaptation implied the production of an specific set of enzymes, which have to be excreted at a determined concentration in the external media. Both, the number and nature of amylolytic activities, and the total starch degrading activity were higher in nitrate containing medium than in ammonium one, optimally supplying all the needs for optimal growing. This ability has been probed vital for the organism to grow in each medium.

Nitrate and ammonium have already been reported to 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, different amount of enzyme excreted to the medium, the different set of enzymes implied in each case may differ depending on this nitrogen source. Their isolation and characterisation are necessary for better understanding of the microorganisms, like in this case, extreme halophilic archaea, which deals with the conditions they have to endure and succeed.

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 characterised by Bautista et al. (2012), while others, regretfully, remain uncharacterised. Assignation and characterization of these putative degrading enzymes is essential to better understanding the mechanisms to get a complete and controlled processing of starch. Amylolytic enzymes are important tools in industry, and specially, to get enzymes specific for each porpoise or application.

In conclusion it can be said that the composition of media strongly determines the set of enzymes employed by the organism to profit starch. Learning more about how the organism gets an optimal profit of starch, implies knowing more about the great variety of amylolytic enzymes present in their genome.

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