**Initial Approaches Towards the Understanding of Catabolite Repression in Halophilic Archaea and the Characterization of an alpha-glucosidase (Maltase) from *Haloquadratum walsbyi***

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A Thesis Submitted in Partial Fulfillment of the
Requiriments for the Degree of
MASTER IN SCIENCE
in
BIOLOGICAL SCIENCE
(Microbiology)
UNIVERSITY OF PUERTO RICO
MAYAGUEZ CAMPUS
2010

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**Abstract**

The work of Carl Woese based on ribosomal RNA sequence analysis from organisms have led scientist to classify life as we know it into three domains. One such domain is the Archaea. There is a lot of interest in studying regulation of gene expression on these microorganisms. There are few reports about gene regulation in halophilic Archaea, especially with genes related to carbohydrate metabolism. Also there are no reports describing the properties of an extremely halophilic alpha glucosidase (maltase) from the Archaea. The purpose of this research project was to study the putative catabolic repression-like system occurring in the extreme halophilic archaea using *Halogeometricum borinquense* and *Haloquadratum walsbyi* as models. To achieve this goal, a set of enzymes involved in carbohydrate metabolism were selected (α-glucosidase, β-glucosidase, and β-galactosidase) and their specific activity levels were determined on different carbon sources supplementing a minimal medium. The experiments revealed that *H. borinquense* prefers glucose as a carbon source, but the three enzymes seem to be fully repressed at this growth condition. These might indicate that *H. borinquense* uses the system of catabolite repression to regulate the genes involved in carbohydrate metabolism. In addition the activity of the three enzymes studied in this work, were markedly dependent on the salt (NaCl or KCl) present in the enzymatic buffer. Also, this project focused on the cloning, expression, purification, and partial characterization of an alpha glucosidase from the extremely halophilic archaeon *H. walsbyi*. The recombinant enzyme showed optimum conditions at 40 $^{0}$C, 15% NaCl (w/v), and pH of 6.0. This study constitutes the first attempt to study gene regulation by catabolite repression in extreme halophilic archaea.

## Resumen

El trabajo de Carl Woese basado en el análisis de secuencias del ARN ribosomal en organismos, ha llevado a clasificar la vida tal como la conocemos en tres dominios, siendo Archaea uno de estos dominios. Hay pocos informes acerca de la regulación génica en arqueas halofílicas, especialmente con los genes relacionados con el metabolismo de carbohidratos. El propósito de este trabajo se ha centrado en el estudio del posible sistema de represión catabólica en la haloarchaea *Halogeometricum borinquense*. Para lograr este objetivo, un conjunto de enzimas que intervienen en el metabolismo de carbohidratos fueron seleccionados (α-glucosidasa, β-glucosidasa, y β-galactosidasa). Sus niveles de actividades específicas se determinaron utilizando diferentes fuentes de carbono en un medio mínimo de sales. Además, la actividad de las tres enzimas estudiadas en este trabajo depende de la sal (NaCl o KCl) utilizada en el amortiguador de ensayos enzimáticos. Por otra parte, todas las alfa glucosidasas caracterizadas hasta el momento corresponden a arqueas hipertermofílicas. No hay reportes en la literatura donde se describa un alfa glucosidasa halofílica (maltasa). La segunda parte de este trabajo se centró en la purificación, expresión y caracterización parcial de la enzima alfa glucosidasa en la arquea halofilica *H. walsbyi*. Esta enzima presentó su nivel óptimo de expresión a $40^0$C, 15% NaCl y pH 6.0. Este estudio representa el primer intento en estudiar el fenómeno de represión catabólica en las arqueas halófilas.

## Dedication

This thesis is dedicated primarily to my husband Rafael Hilerio and my little princess Andrea Carolina, for the love that they give me every day. To my husband for the patience and the support that he rendered me during this work. To my daughter for the beautiful smile and the strong hugs that she gave me every day when I got back home. They are my force and the reason that I have to try to be a better person every day of my life.

To my parents (Carlos A. López and Edith M. Morales), because of them I am the person who I am. Thanks for everything, for believing in me, for staying with me in the worst and in the best moments of my life, for the love, the advices and the dedication that you always showed me.

To my sisters (Ivelisse and Mildred) for being always my best friends, the best of the world.

Thanks mom and Ivy for taking care of Andrea; it was a great help for me in this stage of my educational career.

**Acknowledgments**

I will like to thank God for the life that I have, my husband and little daughter for their support and their love. Thanks to my parents and sisters for believing in me.

I take this opportunity to express a deep sense of gratitude to my research mentor Dr. Rafael Montalvo, for his constant encouragement and sustained interest throughout the course of this research project. Especially for helping me to reach this goal and for being part of my knowledge enrichment in the area of biological sciences. Thanks for believing in me and for this great opportunity.

I would like to thank Dr. Carlos Rodríguez Minguela and Dr. Juan Carlos Martínez Cruzado, for accepting being part of my graduate committee and for being an active part in this project.

I deeply appreciate the help and support given to me by Magaly Zapata. Thanks for the technical assistance and all the favors you did for me during this work. Thanks for your friendship and for being one of my instructors and mentors in the microbiology laboratoty.

I want thank to all my undergraduate students of the Biology Department for the great help that all of you provided me during this investigation. To Jennifer Lopez,

Judith Ventura, Mariola Rivera, Tatiana Meléndez and Grecia Pérez. Thanks for your friendship and support.

I will like to thank all my students from the biological sciences and microbiology courses for being part of my professional goal as an educator in this field of science.

I deeply appreciate the support that all of the members of the Extremophiles Lab gave me over the years. To all of my companions for your cooperation, friendship and lived moments.

Especially I would like to thanks my friends: Zenaida, Gina, Lizaury and Odalys for all the joyful moments shared, support, advices and friendship. I feel very fortunate for meeting all of you. Without your friendship, this part of my life would have never been the same.

Finally, thanks to the Dean of Arts and Sciences at the University of Puerto Rico-Mayaguez Campus, the University of Puerto Rico Biology Department and NASA Space Grant for financially supporting my project.

## Table of Contents

## List of Figures

## List of tables

# Chapter 1. Physiological Studies of the Global Regulatory Response of Glycosyl Hydrolase Expression: a Putative Catabolic Repression-like System in the Extreme Halophilic Archaeon *Halogeometricum borinquense*

## General Introduction

The work of Carl Woese based on ribosomal RNA sequence analysis in organisms led scientists to classify life into three domains (Woese et al., 1990). One of such domains is the Archaea, where its members have unique features that make them different. Despite having a prokaryotic cell organization, the Archaea share physiological and molecular processes that are eukaryotic-like in nature. For example the metabolic aspects of the archaeas are related to those seen in Bacteria; in contrast the mechanisms involved in information processing are more similar to those in Eucarya (Zlatacova, 1997; Ouhammouch, 2004).

Archaea must have novel mechanisms to regulate gene expression, especially considering the types of environments on which they thrive. Hence, archaeal gene regulation can be used as a resource to study microorganisms as new sources of biomolecules and enzymes with biotechnological potential. The stability that these enzymes have at extreme pH, high temperatures, high pressure, high salt concentration, tolerance of organic solvent, and metals, are an advantage for processes performed at harsh conditions in the industrial biotransformation (Egorova et al., 2005). Also, understanding the mechanisms of archaeal gene regulation we can obtain valuable

information about gene expression in eukaryotes. Under this category, genes involved in carbohydrate utilization are of great interest. Carbon catabolite repression (CCR) is a term used to describe how the presence of a carbon source in the medium repress the expression of certain genes and operons, whose products are related with the utilization of alternative carbon sources (Bruckner et al., 2002). Catabolic repression is a paradigm for global and specific gene control mechanisms (Magasanik, 1987). In prokaryotes, catabolic repression in addition with inducer exclusion and transient repression are known as the glucose effect or repression of catabolic enzyme synthesis by glucose (Magasanik, 1987). In bacteria and eukaryotes the system of CCR has been extensively studied, but the existence of this process in archaea, has been poorly investigated (Haseltine et al., 1996). One of the most important studies related with CCR in archaea confirmed the existence of a global gene regulatory system in the hyperthermophilic archaeon *Sulfolubus solfataricus* by Haseltine and coworkers in 1999. Additional studies have supported these conclusions for *S. solfataricus* (Hoang et al., 2004; Lubelska et al., 2006) but the mechanistic details have remained unclear.

There are few reports concerning gene regulation in halophilic Archaea especially with genes related to carbohydrate metabolism. Also there are no reports in the literature describing alpha- glucosidase (maltase) regulation for an extremely halophilic Archaea. Therefore, the main goal of this research project consisted in studying the physiological response of *H. borinquense* cultures grown with different carbon sources by means of glycosyl hydrolase expression. The findings presented in this chapter provide initial evidence of a putative global repression system in response to carbohydrates and are the

first step towards the understanding of catabolic repression in extremely halophilic Archaea.

# Literature Review

## I. Archaea Domain

The Archaea Domain was not recognized as a major domain of life until quite recently. Until 1970, life was classified based on the model of Five Kingdoms divided in two groups: prokaryotic and eukaryotic organisms. This model was shattered in the late of 1970s, when Carl Woese and coworkers demonstrated that life consisted of three distinct lineages (Brown et al., 1997). Based on ribosomal RNA sequence analysis, Woese proposed a formal system of classification where a new taxon called Domain was created. Life on this planet would then be seen as comprised by three domains, the Bacteria, the Archaea and the Eucarya (Woese et al., 1990).

This method of classification places the Archaea in a distinct group of organisms that differed in fundamentals ways from Bacteria and Eucarya. Archaea are considered prokaryotes organisms, because they don't have organelles, cytoskeleton and lack the nuclei; but at the molecular level they are a complex mosaic of features that are prokaryotic or eukaryotic in nature; in addition to their own unique characteristics (Brown et al.,1997 ; Zlatanova, 1997).

The word Archaea comes from the Greek word meaning "ancient", because the hypothesis exists that the Archaea represent the oldest life forms. Few years ago the term Archaea was considered a synonymous to extreme environments. But, the large quantity

of environmental rRNA gene sequences available in databases show that these organisms are present in most of the environments examined to date (Robertson et al., 2005). The Kingdom Archaea is composed of microorganisms that differs in their morphology, physiology and natural habitats (Ouhammouch, 2004). Most of the isolated archaea inhabit some of the most extreme environments in the planet. Such extreme habitats include the deep sea vent with temperatures over 100 $^{0}$C, hot springs, extreme alkaline or acid waters, as well as extremely saline waters. But PCR amplifications of rRNA sequences revealed the presence of these organisms in mesophilic environmentsalso like samples from plant roots, peat lands, freshwater lakes, ocean water, ocean sediments, solid gas hydrates, tidal flat sediments, soil, petroleum-contaminated aquifers and the human mouth and gut (Robertson et al., 2005).

Archaea are divided into four kingdoms: *Euryarchaeota* and *Crenarchaeot*a (which constitute the two major archaeal linages), *Nanoarcaheota* and *Korarchaeota*. Members of the *Euryarchaeota* form a diverse group composed of methanogens, acidophiles, thermophiles and halophiles. Almost all know members of the *Crenarchaeota* inhabit extremely hot environments and in addition are often found to be acidophiles. Its Kingdom includes the most diverse collection of microorganisms (Luo et al., 2001). *Sulfolubus solfataricus* is the most widely studied organism of the crenarchaeal branch and is a model for research on mechanisms of the cell cycle and chromosomal integration, as well as DNA replication, transcription and translation in Archaea. The existence of the kingdom *Korarchaeota* has only been proven by sequence analysis because none of its members has been cultured in the laboratory yet; and the

kingdom *Nanoarchaeota* is represented by only one member *Nanoarchaeum equitans* (a parasite /symbiont of *Ignoccoccus*) (Makarova et al., 2003; Ouhammouch 2004).

**II. Halophilic Archaea**

Inside the Archaea, the kingdom *Euryarchaeota* is represented by the extreme halophilic archaea, methane producers, the thermoplasmatales and some hyperthermophiles. The extremely halophilic archaea live in extremely saline or salty environments. Most species in this category prefer 12-23% (w/v) NaCl solutions, and can survive in saturated solutions with 32% (w/v) or 5.5 M NaCl. Some of the genera that compose this group are: *Halobacterium, Halobaculum, Natrialba, Natrosomonas, Natrarococcus, Haloarcula, Halococus, Haloferax, Halogeometricum, Haloquadratum, and Hm (*Grant and Larsen, 1990; Montalvo-Rodríguez, 1998*).*

Many of the extremely halophilic archaea do not form spores, are gram-negative and reproduce by binary fission. In addition, the halophilic archaea have some of carotenoids pigments that protect the cells from the harmful effects of the ultraviolet radiation and are responsible for the characteristic reddish purple color present in the water were they live (McGenity et al., 2000).

Archaeal enzymes studies are very scarce and ther physiological and biochemical properties are poorly understood. The main problem in fact relies on the stability of these

enzymes under the conditions used. Some studies that deal with the use of halophilic archaeal enzymes are the following: the purification and biochemical characterization of an extracellular serine protease produced by *Natrialba madagii* (Giménez et al., 2000); the characterization of a highly stable α- amylase from the halophilic archaeon *Haloarcula hispanica* (Hutcheon et al., 2005); α-amylase activity from the archaeon *Haloferax mediterranean* (Pérez et al., 2003); and the purification, sequence and expression of the extremely halophilic β-galactosidase from *Haloferax alicantei* (Holmes et al.,1996; Holmes et al.,2000).

**III.Transcription in Archaea: promoter structure and transcription factors**

Archaeal transcription machinery is a mosaic of components from all three domains of life. Although the domains Archaea and Bacteria appear to be very similar in terms of general genomic organization, many archaeal genes show greater similarity to eukaryotic homologs (Brown et al., 1997). Especially, genes encoding fuctions for DNA replication, RNA transcription and protein translation factors, are considered to be more related to those in eukaryotes (Hirata et al., 2009).

Archaeal organisms have a simplified transcriptional apparatus similar to eukaryotic RNAPII system, but much simpler. Eucaryotic organisms use a RNA pol II to initiate transcription of protein coding genes. This RNA pol II consisting of several

subunits, and various basal factors, such as the TATA binding protein (TBP), a component of transcription factor TFIID and transcription factors (TFIIB), (TFIIA),(TFIIE),(TFIIF),and (TFIIH) (Hickey et al., 2002). In contrast, archaeal RNAP seems to only require the following transcription factors: TATA binding protein (TBP), TFB and TFE; homologous to eucaryal's TFIIB and TFIIE (Bartlett, 2005; Bell et al.,2001). Also Archaeal promoters contain the sequence elements (TATA box and B-recognition element (BRE)).

The TATA box is an A/T rich sequence centered at approximately 25 bp upstream of the transcription start site (Ouhammouch, 2004). Like eukaryotic transcription mechanismis, archaeal TBPs are responsible for the recognition of TATA elements. TFB recognized and stabilizes the complex bound DNA-TBP. Often, TFB enhances the sequence specificity of promoter recognition by making additional contacts with the B-recognition element (BRE), located next to the TATA box (Werner et al., 2005). BRE is a purine rich segment that mediates sequence specific interactions with TFB upstream of TATA box. In addition this sequence element directs the machinery of the transcription complex, its assembly, and transcription initiation (Ouhammouch, 2004). TBP-TFB complex, recruit and direct the RNAP to specifically initiate transcription at the initiator sequence. Figure 1 shows the mechanisms in archaeal transcription inititiaton (Bell et al., 2001).

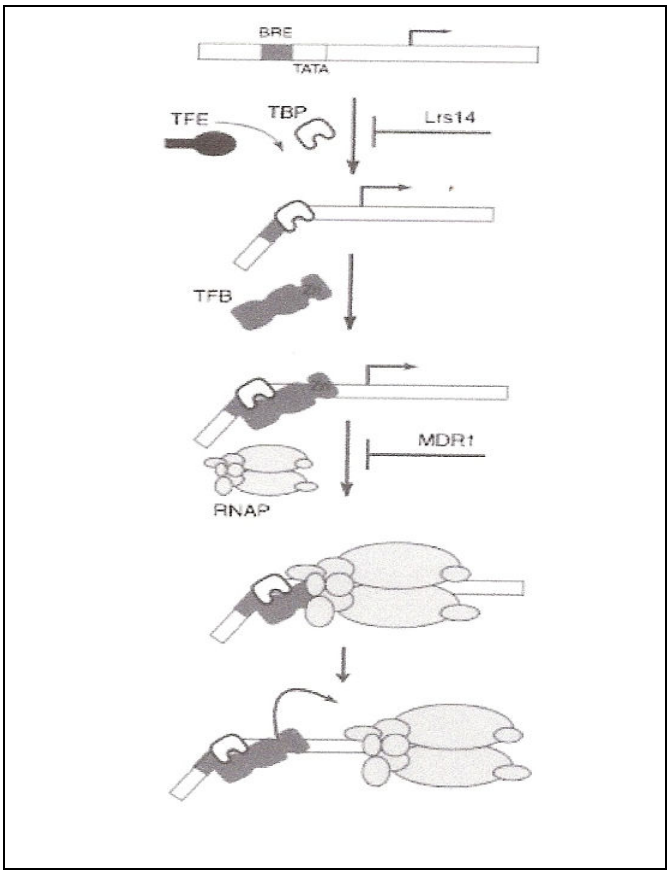


**Fig. 1. Diagram of the steps in archaeal transcription initiation. Taken from Bell et al., 2001.**

## IV. Genetic Expression in Archaea

The regulation of gene expression is one of the topics that are extensively studied for both Bacteria and Eukarya domains. Of the 4,000 genes in the typical bacterial genome, or perhaps 20,000 genes in the human genome, only a fraction is expressed in a cell at any given time. Regulation of gene expression is one of the mechanisms that the cell uses to ensure that those genes necessary at a given moment are expressed at the right

time (e.g. only in the presence of a particular substrate). The expression of genes is regulated by processes that affect the rates at which gene products are synthesized and degraded. Much of this regulation occurs at the level of transcription initiation, mediated by regulatory proteins that either repress transcription or activate transcription at specific promoters (Conaway et al., 2002).

In bacteria, genes that encode interdependent functions are often clustered in an operon. Transcription of the genes is generally blocked by binding a specific repressor protein at a DNA site called the operator. Dissociation of the specific repressor from the operator is mediated by a specific small molecule, and inducer (Struhl, 1999). These principles were first elucidated in studies of the lactose operon. In eukaryotes, positive regulation is more common than negative regulation, and transcription is accompanied by large changes in chromatin structures (Struhl, 1999). Promoters for RNA Pol II typically have a conserved DNA sequence knows as the TATA box, as well as multiple binding sites for DNA-binding transactivators. The latter sites, sometimes located hundreds or thousands of base pairs away from TATA box, include upstream activator sequences in yeast and enhancers in higher eukaryotes (Boube et al., 2002). Large protein complexes are generally required to regulate eukaryotic transcriptional activity. The modular structures of the transactivators have distinct activation and DNA binding-domains. Other protein complexes include histone acetyltranferases and ATP- dependent chromatin remodeling complexes such as SWI/SNF and NURF (Boube et al., 2002; Cosma, 2002).

There is not much information about the Archaeal molecular mechanisms that control gene expression, relative to that described for the Bacteria and Eucarya domains. The knowledge having today has been inferred from the presence of a promoter and transcription machinery similar to those seen in eucaryotes (Kuo et al., 1997). Transcription in Archaea is catalyzed by an RNA polymerase homologus to eukaryiotic RNAP II with similar subunit composition and transcription factors. The archaea have a TATA box, TBP, some transcription factors and also have introns in the tRNA genes that do not exist in bacteria. Also archaea have proteins associated with their DNA very similar to the histone proteins of the eukaryotes. These discoveries suggest that archaea and eucaryal transcription systems are related, but little is know about the mechanism responsible for gene regulation in members of the domain Archaea (Kuo et al., 1997).

The present of gene regulatory systems have been found in members of *Euryarchaeota* kingdom. Examples including the regulation of synthesis of bacteriohododpsin, halocins, gas vesicles and the heat shock response, all presents in halophilic arcahea (Haseltine et al., 1999). However, little is known about gene regulation in halophilic archaea with respect to genes involved in carbohydrate metabolism.

**V. Catabolite repression in archaea**

The mechanism of switching on and off the lactose operon has been described in detail by Jacob and Monod in 1960 and termed ‘glucose effect’. This phenomenon was

termed ‘catabolite represssion’ when studies reflect the present of this system involved in the metabolism of other carbon sources. Carbon catabolite control is a mechanism used by the cell to coordinates the metabolism of carbon and energy sources and maximize its efficiency (Fujita, 2009). Carbon catabolite repression (CCR) is regulatory mechanisms in wich the presence of a preferred substrate repress the expression of the genes required for the utilization of secondary sources of carbon. Gene regulatory strategies used to accomplish catabolite repression include both positive and negative mechanisms.

Many studies have shown that bacteria and eukaryotes regulate the expression of genes involved in carbohydrate utilization using transcriptional regulatory mechanisms (Haseltine et al., 1999). In contrast little is known about CCR in archaea, especially halophilic archaea. One of the few reports of the existence of a global gene regulatory system implicated in carbohydrate utilization was described in the hyperthermophilc arcahaeon *Sulfolobus solfataricus* (Haseltine et al., 1999). *S. solfataricu*s became a model organism to study replication, transcription, translation and gene expression regulation for crenarchaeotes, and more generally in Archaea. The study performed by Haseltine and coworkers indicated that this organism employs a global transcriptional regulatory system to coordinate its response to carbon sources. The system exhibits features found previously in the catabolite repression systems of eukaryotes and bacteria such as coordinated regulation of expression of genes involved in the metabolism of secondary carbon and energy sources; but certain aspects such as inducer exclusion and transient repression are not apparent (Hasseltine et al., 1999). This system controls expression of several glycosyl hydrolases, including *malA* (α-glucosidase), *amy*A (α-amylase), and

*lac*S (β-glycosidase), at the level of transcript abundance (Haseltine et al., 1999). The activities of the α-glucosidase, β-glycosidase, and α-amylase, responded to the presence of supplementary carbon sources such as amino acids (Haseltine et al., 1999). Catabolite repression has also been studied extensively in *Pyrococcus*. The expression of the genes related with the metabolism of β-glucosidase and two alcohol dehydrogenases has been studied, and results revealed the presence of a coordinated regulation system and adaptation of *Pyrococcus* to different carbon sources (Lubelska et al., 2006).

## VI. *Halogeometricum borinquense*: the extremely halophilic archaeon under study

*Halogeometricum borinquense* is an extremely halophilic archaeon isolated and characterized from the solar salterns of Cabo Rojo, Puerto Rico by Montalvo-Rodríguez et al., in 1998. This microorganism is aerobic, chemeorganotrophic, motile (peritrichous flagella) gram negative and presents pleomorphic shapes (short and long rods, squares, triangles, ovals) when grown on Shegal-Gibbons broth (Montalvo-Rodríguez et al., 1998) (Fig. 2). This organism requires at least 8% NaCl (w/v) to grow, but optimal growth is achieved at 40 $^{0}$C, pH 7 and 20-25% NaCl (w/v). After one week of incubation in solid medium, it presents pink and convex colonies with circular shape, and entire margin (Fig. 2). In addition this organism can use glucose, mannose, fructose, xylose, maltose, trehalose, cellobiose, raffinose and glycerol as carbon sources (Montalvo-Rodríguez et al., 1998).

a. b.

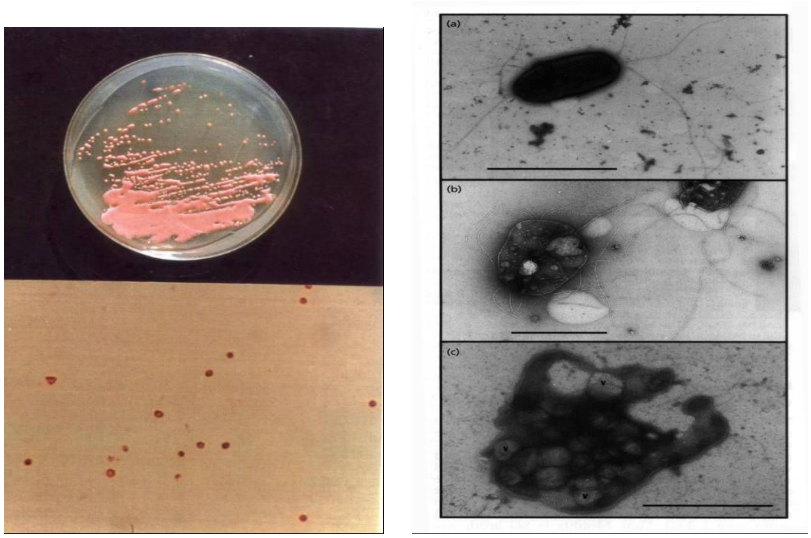


**Fig. 2. a. Morphological and cell characteristics of *H. borinquense* cultures. b. Scanning Electron Microscopy of *H. borinquense.* (taken from Montalvo-Rodriguez et al., 1998)**.

## Materials and methods

### Archaeal strain and cultivation

*Halogeometricum borinquense* (ATCC 700274) was grown in Shegal Gibbon's medium (Nicolaus et al., 1999). The medium consists of (g/L) trisodium citrate 3.0, KCl 2.0; MgSO4 $\bullet 7H_2O$ 20; NaCl 200; $FeCl_3$ 2.3 μg; yeast extract 10; casamino acids 7.5. The pH was adjusted to 7.2-7.8 with 1M NaOH, and autoclaved at $121^0$C for 20 minutes. Cells were cultured in 250-ml Erlenmeyer flask containing 50 ml of medium, and incubated at $40^0$C on a rotary shaker at 100 rpm. Growth was monitored spectrophotometrically at a wavelength of 540 nm.

### Determining the optimum amount of yeast extract

To determine the minimal amount of yeast extract needed as nitrogen source, *H. borinquense* was grown in a minimal salts medium with differents concentrations of yeast extract (.01%, .05%, .1%, 1%) without any carbohydrate as carbon source. The minimal salts medium consist of (g/L) trisodium citrate 3.0, KCl 2.0; MgSO4 $\bullet 7H_2O$ 20; NaCl 200; $FeCl_3$ 2.3 μg. The pH was adjusted to 7.2-7.8 with 1 M NaOH and autoclaved at $121^0$C for 20 minutes. Cells were cultured in 250-ml Erlenmeyer flask containing 50 ml of medium, and incubated at $40^0$C on a rotary shaker at 100 rpm. Growth was monitored spectrophotometrically at a wavelength of 540 nm.

**Determination of optimal conditions for maltose utilization and measurement of α-glucosidase activity**

Optimal conditions for maltose utilization were determined by growing *H. borinquense* in a minimal salts broth (20% (w/v) NaCl, using different yeast extract concentrations as nitrogen source (0.01%, 0.05%, 0.1% and 1%), supplemented with 0.2% (w/v), 0.4% (w/v) and 0.6% (w/v) of maltose as carbon source at 40 $^0$C. The cells were cultivated by constant agitation (100 rpm), until the culture reach a late log phase of growth. Its was monitored by measuring absorbance at 540 nm. All conditions were performed in duplicate.

To determine the α-glucosidase activity and whether it might be a secreted extracellular or an intracellular enzyme , 50 ml of the culture broth were centrifuged at 5,000 rpm for 15 minutes, and the cell free supernatant was collected. The pellet (which contains the cells) was resuspended in 1ml 50mM Tris HCl, 3M NaCl, pH 7.2 and sonicated five times for 1 minute each. The cell extract and supernatant were analyzed to determine the location of the enzyme; the assays were performed at different temperatures (37 $^0$C, 40 $^0$C and 50 $^0$C). All the assays were performed in duplicates.

**Physiological studies, growth kinetics effects of carbon source in glycosyl hydrolase activity**

Various carbon sources (glucose 0.4% (w/v), sucrose 0.4% (w/v) and maltose 0.6% (w/v)) were used as supplements of a minimal salts medium to study their effect on

the expression of some glycosyl hydrolases (α-glucosidase and β-glycosidase and β-galactosidase). Growth curves and enzymatic assays were performed under these conditions in duplicate.

**Enzyme assays**

Assays for α-glucosidase, β-glycosidase, and β-galactosidase were performed using *H. borinquense* cell extracts prepared by sonicating cells resuspended in 50mM Tris-HCL with 3M NaCl (pH 7.2), and in 50mM Tris-HCl with 3M KCl (pH 7.2). The hydrolysis of p-nitrophenyl-α-glucopyranoside (α-PNPG) was used to measure α-glucosidase activity as described previously (Rolfsmeier and Blum, 1995). This substrate was added to a reaction buffer consisting of 50mM Tris hydrochloride, 3M NaCl; (pH 7.2). Reactions were initiated by the addition of crude cell extract or supernatant to the substrate solutions and terminated by the addition of 500 µl 1M sodium carbonate. The same procedure was repeated using Tris HCl 50mM, 3M KCl as the enzymatic buffer. Hydrolysis of β-PNPG and θ-PNPG was used to determine the β-glycosidase and β-galactosidase activity, using the same procedure described for the α-glucosidase. The extent of substrate hydrolysis was determined by the absorbance of the sample at a wavelength of 420 nm. Assays containing only the substrate or samples from the cells extract and supernatant were used as control to connect for background signals. Each experiment was performed in triplicate. A unit of either α-glucosidase and β-glycosidase activity is defined as the amount of enzyme required to liberate 1 µmol of ρ-nitrophenol per min per mg of total protein.

**Protein assays**

Protein concentration was determined using the Pierce BCA Protein Assay kit as described by the manufacturer, using bovine serum albumin (BSA) as a standard.

## Results

### Optimal conditions for growth and enzyme production

Since there are no reports in the literature where optimal growth conditions for utilization of a sole carbon source are described for *H. borinquense*, growth kinetics experiments were performed to determine such conditions. Halophilic archaea need organic nitrogen for growth (Bonete et al., 2008), therefore reconstruction experiments were performed to determine the amount of yeast extract necessary to be a nitrogen source and not a carbon source in the medium. Growth kinetic experiments were performed on *H. borinquense* using different concentrations of yeast extract (0.01%, 0.05%, 0.1, and 1% w/v). Figure 3 shows that using yeast extract at 0.01, 0.05 and 0.1% does not provide enough carbon (2 doublings or less) resulting in lower yields of growth. Using 1% of yeast extract provide an excess of organic carbon that are a sufficient amount to promote a good growth; this issue interfered with source of carbon used in the kinetics growth experiments. Results showed that decreasing the level below 0.1% did not present a significantly influence, but biomass was found to be greatly increased when the level of yeast extract was 1%.

The behavior of *H. borinquense* was studied at different concentrations of maltose as a sole carbon source (0.2% , 0.4% , 0.6% ) in a minimal salts medium containing 20% (w/v) NaCl and several concentrations of yeast extract (0.01, 0.05%, 0.1%). This experiment was performed in order to determine the best combination of carbon and

nitrogen source for *H. borinquense* growth. *H. borinquense* did not grow when .01% of yeast extract was used as nitrogen source, with any of the concentrations of maltose as carbon source. These results demonstrate that 0.01% of yeast extract as nitrogen source is not sufficient to support growth of *H. borinquense*. In contrast *H. borinquense* presented the highest growth yields when .05%(w/v) and 0.1%(w/v) of yeast extract were used as nitrogen source and 0.6% (w/v) of maltose as a carbon source. Since there are not significant differences between .05% and 0.1% of yeast extract as nitrogen source in the medium, we decided to use 0.1% as the optimal yeast extract concentration to have an excess of nitrogen.

Figure 4 summarizes the data described above, and presents the behavior of *H. borinquense* when growth in salts minimal medium containing 20% NaCl(w/v), 0.1% of yeast extract and different concentrations of maltose as a carbon source (0.2%, 0.4%, and 0.6%.). The highest growth yield was observed for the combination of 0.1% of yeast extract and 0.6 % of maltose (5 doublings). This combination of conditions has the shortest generation time (49.3 h) (Table 1). Figure 5 confirms the use of maltose as carbon source and proves that 0.1% of yeast extract is used only as a nitrogen source.

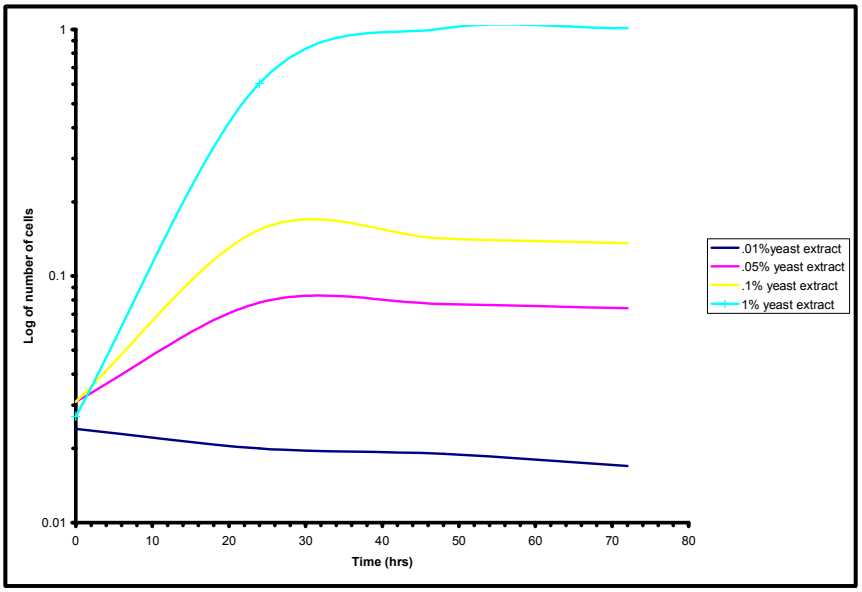


**Fig. 3. Growth kinetics of *Halogeometricum borinquense* grown in a minimal medium containing: 20%(w/v) NaCl and different concentrations of yeast extract (.01%,.05%,.1%,1%) as organic nitrogen.**

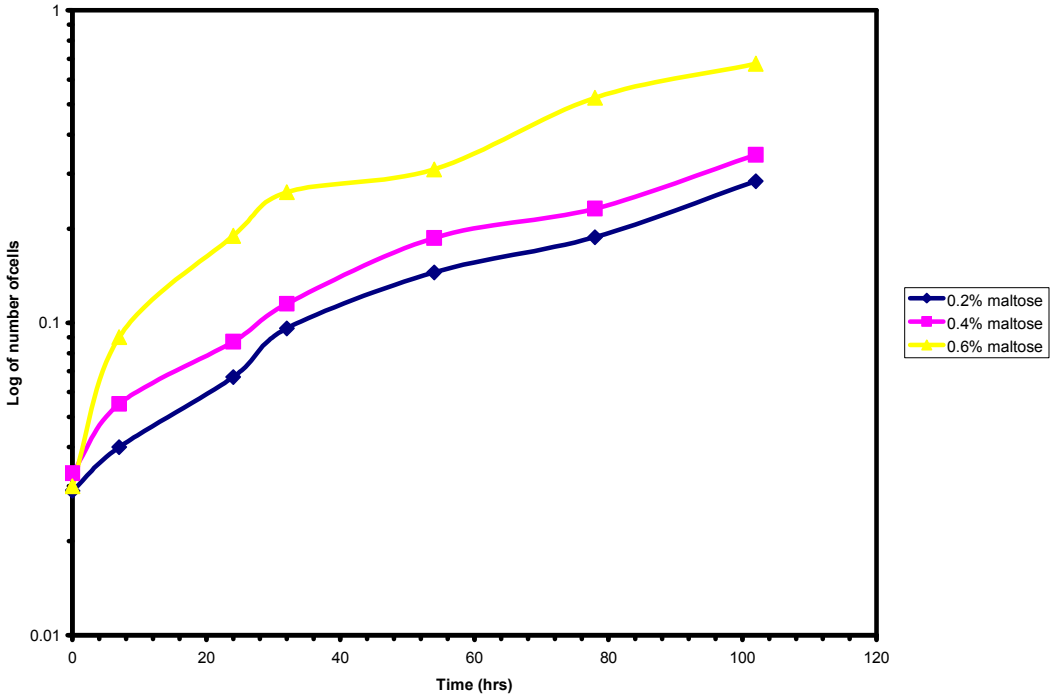


**Fig. 4. Growth kinetics of *Halogeometricum borinquense* growth curves in minimal medium containing 20%NaCl, , 0.1% (w/v) yeast extract and different concentrations of maltose (0.02% , 0.4 %, 0.6%).**

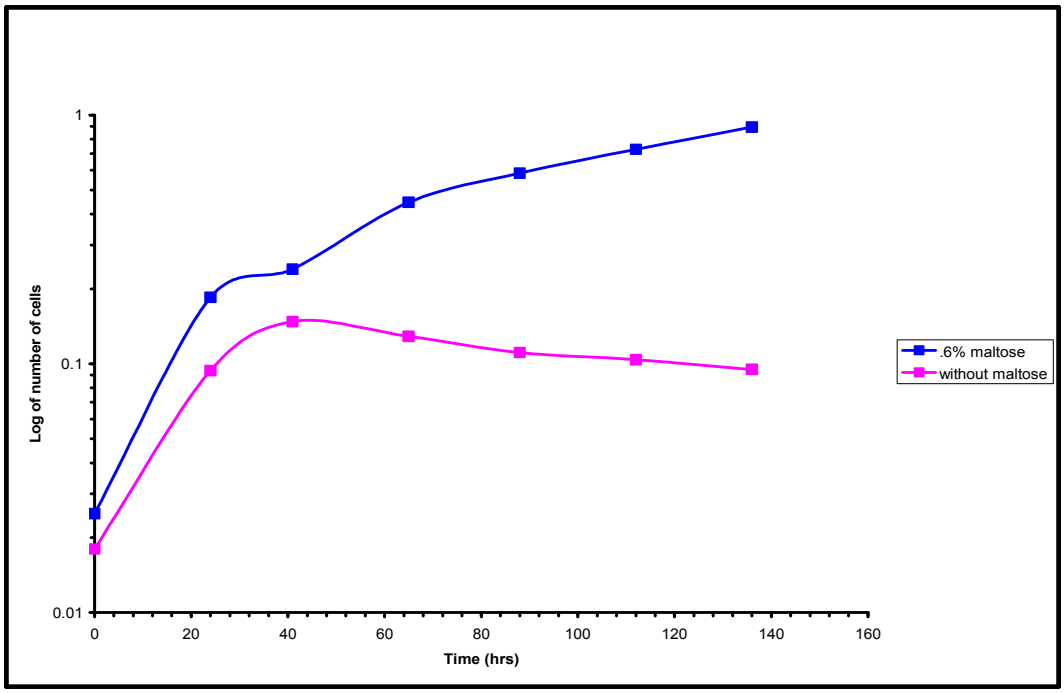


**Fig. 5. Growth kinetics of *Halogeometricum borinquense* growth curves. S.G minimum medium 20% NaCl; 0.1% yeast extract; with maltose as carbon source and without maltose.**

**Table 1. Generations times of *H. borinquense* during growth in minimal salts medium (20% (w/v) NaCl and 0.1% (w/v) yeast extract) on different concentration of maltose as a carbon source**

| Matose(w/v) | Generation Time (h): |
|---|---|
| 0.2% | 125.4 |
| 0.4% | 103.7 |
| 0.6% | 49.3 |

**Measurement of α-glucosidase activity**

To check whether the α-glucosidase activity is an extracellular or intracellular proteins, enzyme activity assays were performed for the analyses of the pellet and the supernatant fractions of *H. borinquense* cultures (S.G 20% NaCL, 0.1% (w/v) yeast extract, and 0.6% (w/v) maltose). All the enzymatic assays were performed using cultures with maltose (0.6% (w/v) and without maltose. The cultures used were at a late log-phase of growth. In addition the enzymatic assays were performed at different temperatures ($37^0$C, $40^o$C, $50^o$C), to determine the optimum temperature of the enzyme (Table 2). Assays were performed using p-nitrophenyl α-D glucopyranoside (PNPG) as the substrate. Triplicates of all the enzymatic assays were performed. No activity was shown when the supernatant was used as the enzymatic extract, for any of the assays performed at the different temperatures; also no activity was detected for any of the cultures without maltose.

**Table 2. Optimum temperature and distribution of the α-glucosidase enzyme of *H. borinquense***

| | α-Glucosidase Specific Activity (µmoles PNP/ min/mg) Cultures with 0.6% of maltose | |
|---|---|---|
| **Temperature** | **Cell extract** | **Supernatant** |
| 37 $^0$C | 2.7 | N.D. |
| 40 $^0$C | 3.6 | N.D |
| 50 $^0$C | 3.2 | N.D |

***ND= No detected**

**Growth curves of *H. borinquense* in S.G minimal medium with different carbon sources**

Growth curves were independently performed using glucose (0.4% (w/v)), sucrose (0.4% w/v) and maltose (0.6% w/v) as carbon sources (Fig. 6). Cultures achieved at a late log phase were harvested and enzymatic assays were performed to study the physiological response of *H. borinquense* to different carbon sources. Table 3 demonstrates the generation time at different carbon sources (glucose 0.4% (w.v), sucrose 0.4% (w/v), maltose 0.6% (w/v)).

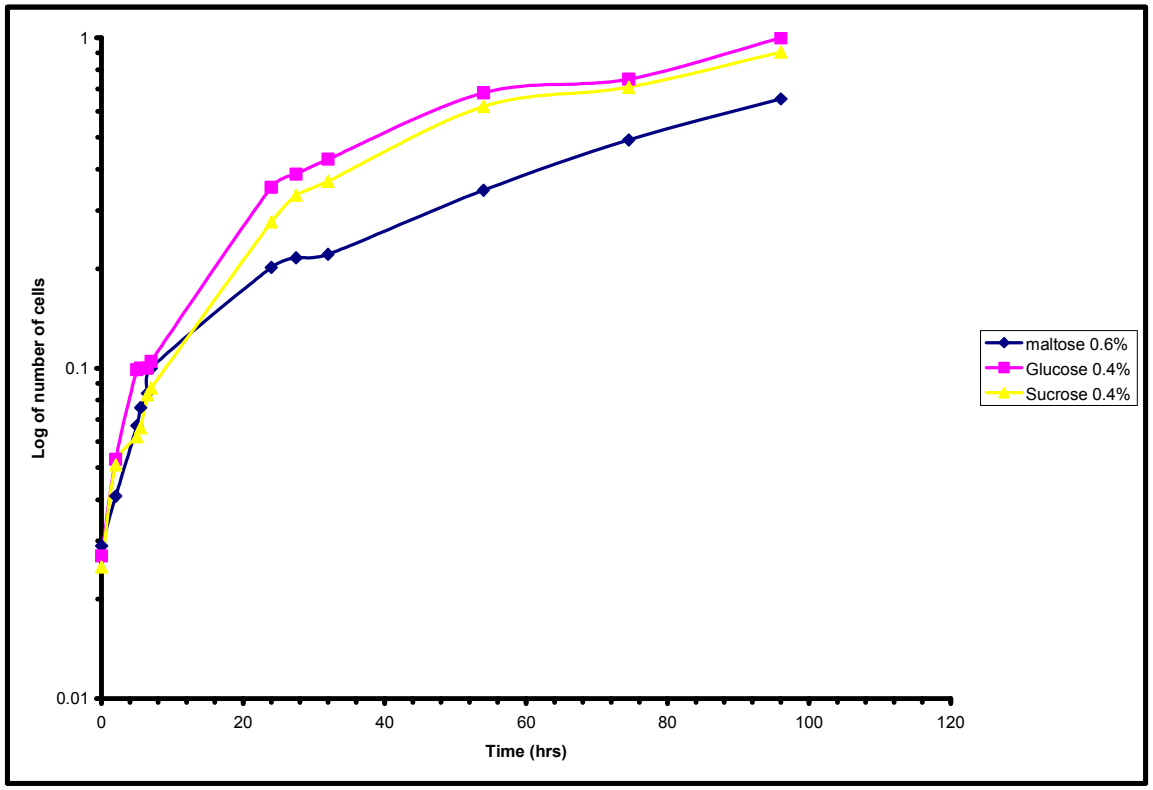


**Fig. 6. Growth curves of *H. borinquense* in S.G minimal medium (20% NaCl, 0.1% yeast extract) with different carbon sources (glucose 0.4% (w/v), sucrose 0.4% (w/v), and maltose 0.6% (w/v).)**

**Table 3. Generations times of *H. borinquense* during growth on different carbon sources.**

| Carbon source | Generation Time (h) : |
|---|---|
| Glucose 0.4% (w/v) | 29.8 |
| Sucrose 0.4% (w/v) | 32.0 |
| Maltose 0.6% (w/v) | 48.5 |

**Effect of carbon sources on glycosyl hidrolases production and effect of different salt buffer on α-glucosidase, β-glycosidase and β-galactosidase activity**

*H. borinquense* was grown in Shegall Gibbons (S.G) rich medium 20 % NaCl and in salts minimal medium (20% NaCl; 0.1%(w.v)) with different carbon sources (glucose 0.4% (w.v), sucrose 0.4% (w/v) and maltose 0.6% (w/v)). Enzymatic assays were performed for each of the cultures with different carbon source. The following substrates: p-nitrophenyl-α-glucopyranoside (α-PNPG); p-nitropheny-β-glucopyranoside (β-PNPG) and o-nitrophenyl-β-D-galactopyranoside (o-PNPG) were used, to study the behavior of the α-glucosidase, β-glycosidase and β-galactosidase enzymes in response to the different carbon source. Each enzymatic assay was performed with two different buffers (Tris HCl 50mM, 3M NaCl and Tris HCl 50mM, 3M,KCl) to see if the enzymes responded differently to a particular salt. All the samples were assayed in triplicate, and the averages results are reported (Figs. 7-12).

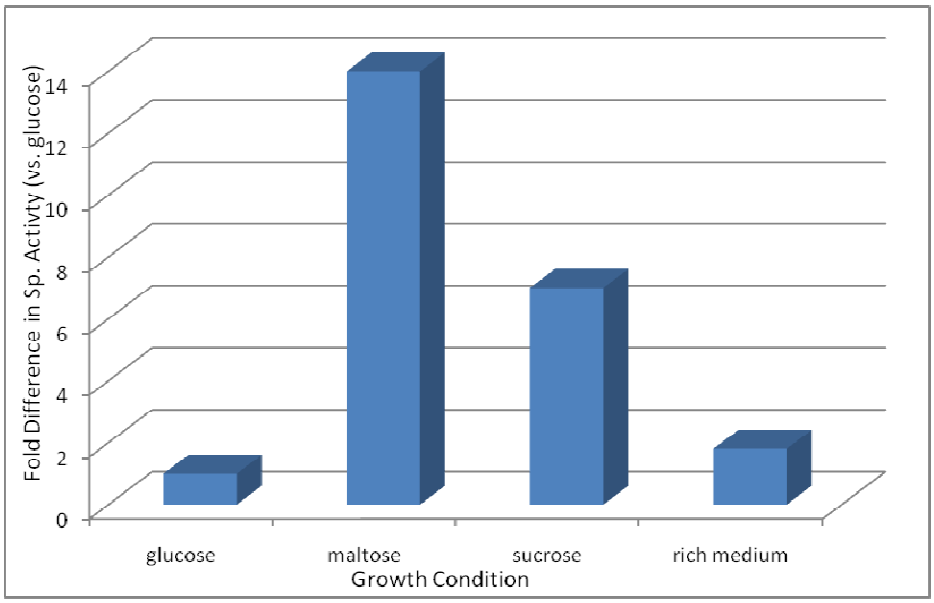


Fig. 7. Fold difference in specific activity of α- glucosidase enzyme with respect to the carbon source. The assays were performed using Tris HCl 50mM, 3M NaCl as the enzymatic assay's buffer and p-nitrophenyl-α-glucopyranoside (α-PNPG) as the substrate.

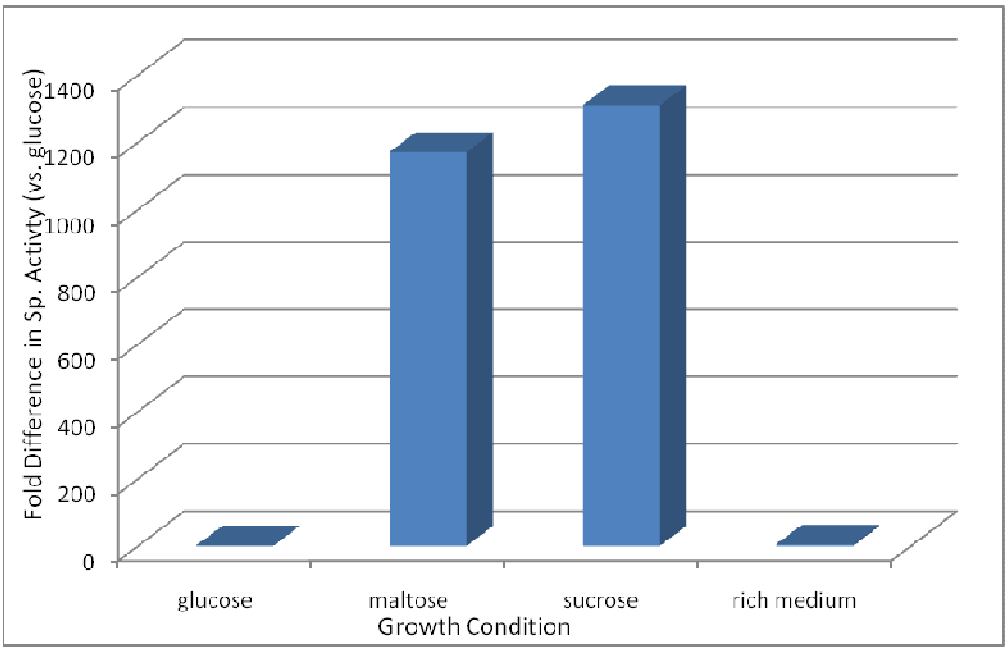


Fig. 8. Fold difference in specific activity of α- glucosidase enzyme with respect to the carbon source. The assays were performed using Tris HCl 50mM, 3M KCL as the enzymatic assay's buffer and p-nitrophenyl-α-glucopyranoside (α-PNPG) as the substrate

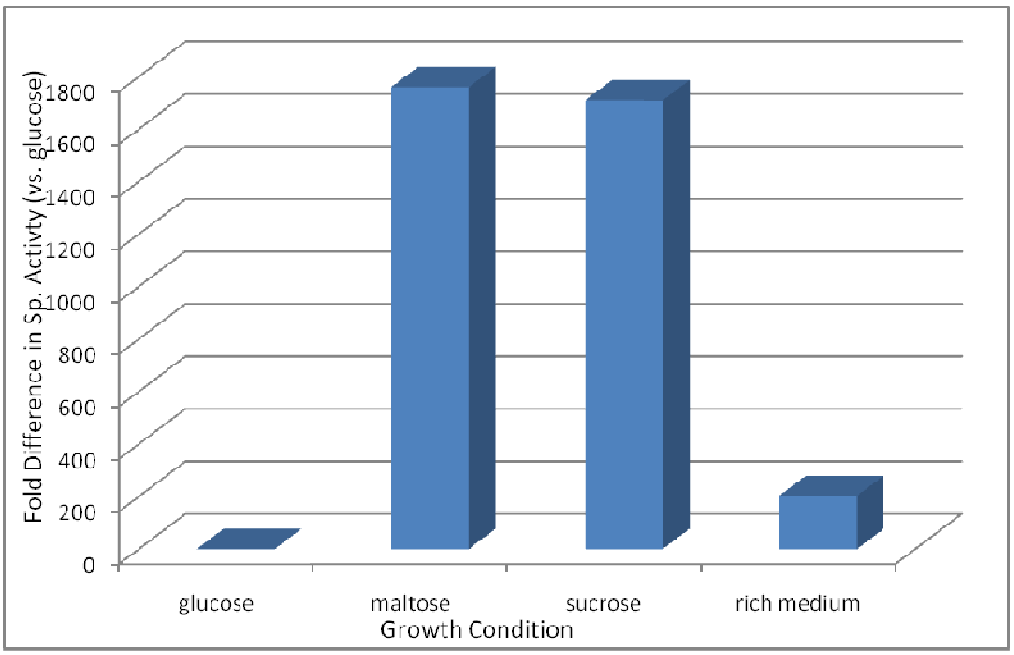


Fig. 9. Fold difference in specific activity of β-glycosidase enzyme with respect to the carbon source. The assays were performed using Tris HCl 50mM, 3M NaCl as the enzymatic assay's buffer and p-nitropheny-β-glucopyranoside (β-PNPG)as the substrate

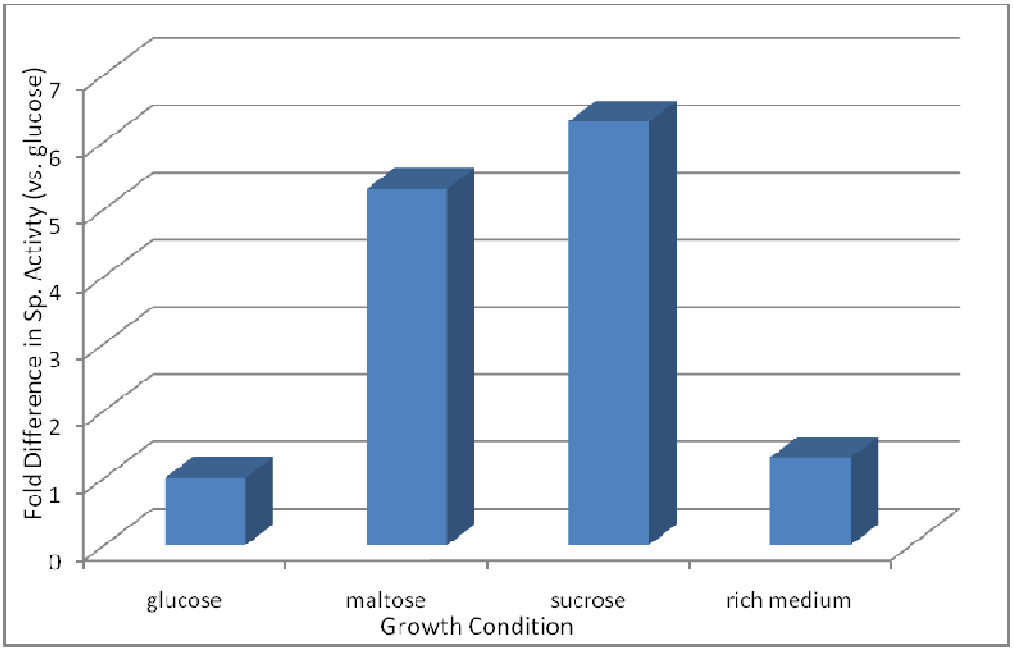


Fig.10. Fold difference in specific activity of β-glycosidase enzyme with respect to the carbon source. The assays were performed using Tris HCl 50mM, 3M KCl as the enzymatic assay's buffer and p-nitropheny-β-glucopyranoside (β-PNPG)as the substrate

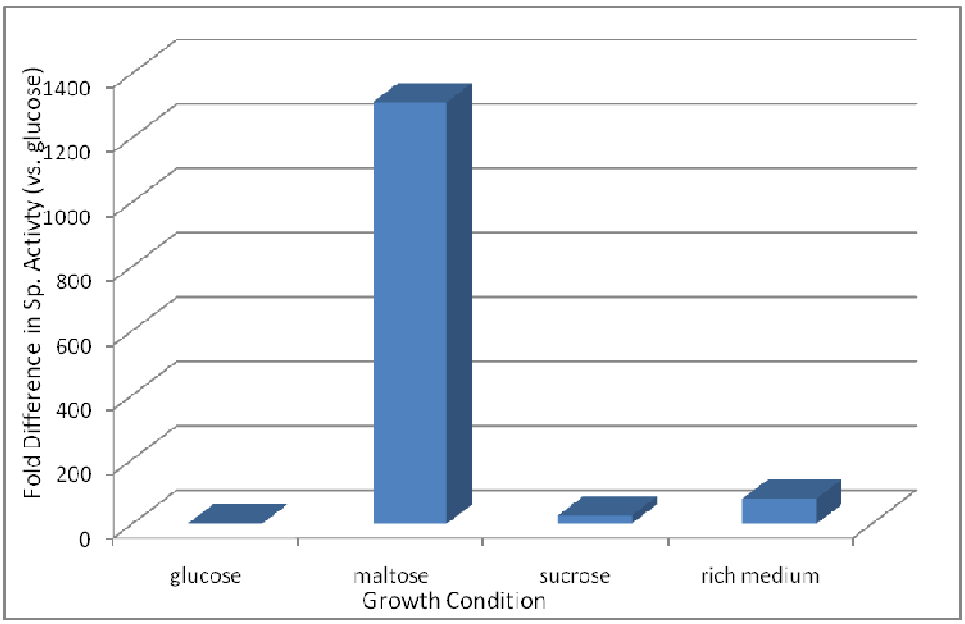


Fig.11. Fold difference in specific activity of β-galactosidase enzyme with respect to the carbon source. The assays were performed using Tris HCl 50mM, 3M NaCl as the enzymatic assay's buffer and nitrophenyl-β-D-galactopyranoside (o-PNPG) as the substrate.

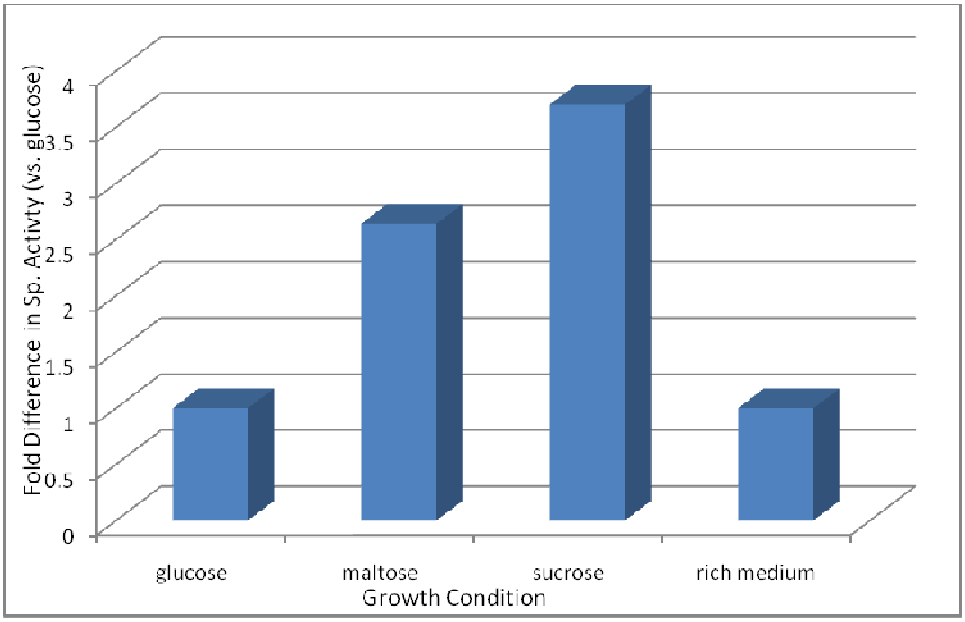


Fig.12. Fold difference in specific activity of β-galactosidase enzyme with respect to the carbon source of. The assays were performed using Tris HCl 50mM, 3M KCl as the enzymatic assay's buffer and nitrophenyl-β-D-galactopyranoside (o-PNPG) as the substrate.

**Discussion**

Regulation of gene expression is one of the mechanisms that cells use to ensure that necessary enzymes are produced at the right time. There are few reports concerning gene regulation in halophilic Archaea, especially with genes related to carbohydrate metabolism. Also there are no reports in the literature describing an extremely halophilic alpha- glucosidase (maltase). Most of the studies performed on α-glucosidases from archaea have focused their objectives on the purification, characterization, and identification of the corresponding genes. There are few reports that address the question of how these enzymes are regulated, or what is the mechanism that controls the expression of their structural genes. *H. borinquense* was selected as the model to study and better understand the physiological response of glycosyl hydrolase expression (including the alpha-glucosidase) of halophilic archaea in cultures with different carbon sources.

Experiments to determine the effect of nitrogen and maltose concentrations revealed that 0.6% (w/v) of maltose and 0.1% (w/v) of yeast extract as carbon and nitrogen sources respectively produced optimal growth yield (five doublings) and alpha glucosidase production for *H. borinquense* in salts minimal medium. Different enzymatic assays were performed in cultures with and lacking maltose. No alpha-glucosidase activity was detected in cultures without a carbohydrate as a carbon source. The results indicate that this is an intracellular enzyme because no alpha glucosidase activity was detected when the culture supernatant was used in enzymatic assays.

Carbon catabolite control is a regulatory mechanism by which cells coordinate the metabolism of carbon and energy sources to maximize its efficiency and to regulate other metabolic process as well (Fujita., 2009). Carbon catabolite repression (CCR) in microorganisms is generally regarded as a regulatory mechanism to ensure sequential utilization of carbohydrates. CCR is a term used to describe the general phenomenon in microorganisms whereby the presence of a carbon source in the medium can repress expression of certain genes and operons, whose genes products are often concerned with the utilization of alternative carbon sources (Bruckner et al., 2002). Three enzymes were selected to help define a putative catabolic regulation-like system in *H. borinquense*: alpha-glucosidase, beta-glycosidase and beta-galactosidase. Activities of each of the three enzymes were determined from cells in the late exponential phase of growth in a minimum media supplemented with 0.1% of yeast extract and three different carbon sources (glucose, sucrose, and maltose). *H. borinquense* utilizes all of these three carbon sources with various efficiencies, as indicated by their respective generation times (Table 3) and growth yields. Growth kinetics performed for *H. borinquense* in S.G medium with these carbon sources indicated that this halophilic archaea prefer glucose as a primary carbon source (near to 6 doublings); sucrose (five doublings) is in second preference and finally maltose (four doublings) (Fig. 6). These results are similar to the majority of documented cases, where the preferred carbon source is glucose with the *Escherichia coli* glucose-lactose diauxie as the classical example (Bruckner et al., 2002). Already in the early sixties, it was observed that *E. coli* prefers glucose over other potential carbon sources. The presence of glucose in the medium prevents the expression of genes involved in the utilization of other carbon source like lactose. Catabolite

repression allows microorganisms to adapt quickly to a preferred carbon and energy source. Recently, the phenomenom of catabolite repression was also reported in the hyperthermophilic archaeon *Sulfolobus solfataricus* (Haseltine et al; 1999). Coordinated regulation of expression of the genes involved in carbon and energy metabolism has been shown; and the activities of α-glucosidases, β-glycosidase, and α-amylase, encoded by *mal*A, *lac*S and *amy*A genes responded to the presence of supplementary carbon sources such as amino acids (Hasseltine et al., 1999; Hoang et al., 2004).

Even though *H. borinquense* prefers glucose as carbon source, the enzymes seems to be fully repressed at this growth condition. One of the hallmarks of catabolite repression is the variation in levels of glycosyl hydrolases, in response to a carbon source type (Haseltine el al., 1999). These results might indicate that the halophilic archaea *H. borinquense,* like *E. coli* and *S. solfataricus,* uses the system of catabolite repression to regulate the genes involved in carbohydrate metabolism. When glucose is available in the enviroment, the synthesis of alpha-glucosidase, beta-glycosidase and beta-galactosidase are under repression due to the effect of catabolite repression caused by glucose. When NaCl was used as the enzymatic salt buffer, alpha-glucosidase activity was more than tenfold higher during growth on maltose and sevenfold higher in sucrose than in glucose. Similar results were observed with the other two enzymes studied in this project. The activity for the beta-glycosidase and beta-galactosidase are fully repressed when glucose is used as a carbon source indenpendently of the salt source (NaCl or KCl) used in the enzymatic assay buffer. These results indicate that the observed change in glycosyl hydrolase expression is related to the carbon source present in the media.

Independently of the salt source all the enzymes studied in this work are represed when glucose is present in the medium.

Most of the halophilic archaea grow at salt concentrations above 10%. Adaptations to this harsh environment include maintaining high internal salt concentrations, up to 5M KCl (Holmes et al., 1996). A decrease in the salts required by halophilic enzymes may lead to the loss of their structure and function, and they need high salt concentration for stability and optimal activity (Pérez et al., 2003). Additionally, these enzymes can be either intracellular, extracellular, or membrane bound (Constantino et al., 1990). The activity (μmoles PNP/ min/mg) of *H. borinquense* α-glucosidase in Tris HCl 50 mM- 3M NaCl versus Tris HCL 50 mM- 3M KCl represents a 45.6% difference in activity. Based on these preliminary experiments, we inferred that *H. borinquense's* alpha glucosidase is a membrane bound enzyme. These conclusions were based in the preference of the enzyme for Na+ as the stabilizing ion. On the other hand, the results show that the β-glucosidase and β-galactosidase enzymes could be intracellular proteins. These conclusions were inferred based on the preference of these enzymes for K+ as the stabilizing ion.

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The results presented in this chapter, represent the first report of catabolite repression in the extremely halophilic archaea *H. borinquense*, and describe initial physiological experiments concerning of a regulation of a halophilic α-glucosidase. Additional experiments are needed to determine the source of regulation.

**Chapter 2: Partial characterization of an alpha glucosidase from the extremely halophilic archaea *Haloquadratum walsbyi*.**

**General Introduction:**

Carbohydrates are not only the most abundant carbon source in the biosphere, but also versatile and vitals elements in biological systems. Living organisms use oligosaccharides and polysaccharides for a multitude of biological functions, from storage and structure to high specific signaling roles. A glycosidic bond is the linkage between two or more monosaccharides or between one monosaccharide and other non carbohydrate moieties. The hydrolysis of glycosidic bonds is very important for energy uptake, cell wall expansion, degradation and turnover of signaling molecules (Davies et al., 1995). As a consequence of the great diversity of sacharides there are a large number of enzymes that hydrolyze glycosidic bonds. These enzymes are classified by the Enzyme Commision, as glycolsyl hydrolases (EC 3.2.1.x).

Glycoside hydrolases are a widespread group of carbohydrate active enzymes present in virtually all organisms and these enzymes are responsible for the hydrolysis and the biosynthesis of glycosidic bonds between carbohydrates or between carbohydrate and non carbohydrate moieties (Henrissat, 1991). β-galactosidase, invertase, maltase, α-galactosidase, amylase, are some examples of enzymes that catalyzes the hydrolysis of glycosidic bonds. These enzymes are widely distributed in nature and their existence has been known for more than 100 years; also they were the first biological catalysts

investigated (Gote, 2004). Glycoside hydrolases have different important functions like digestion and decomposition of polysaccharides and biosynthesis of glycoproteins (Lovering et al., 2005). Gene sequences of many of these important enzymes have been now determined, and the corresponding enzymes have been grouped into families on the basis of sequence similarity. Glycoside hydrolases from various sources, including bacteria, fungi, plants and animals were classified into 115 families based on amino acid sequences (Database, Carbohydrate Active Enzymes).

α-Glucosidases (EC. 3.2.1.20) constitute a widespread group of exo-acting glucoside hydrolases that catalyze the hydrolysis of α-D-glucose from the non reducing ends of various α-linked substrates (Alarico et al., 2008; Naested et al., 2006). Alpha glucosidases also catalyze transglucosylation reactions that are used in the biotechnological industry to conjugate sugars with biologically useful materials or to produce food oligosaccharides (Zhoug et al., 2009). These enzymes are important in carbohydrate metabolism, in glycoprotein processing and quality control (Ernst et al., 2006).

All of the alpha glucosidases characterized from archaea correspond to hypertermophilic archaea. There are no reports in the literature describing an extremely halophilic alpha glucosidase (maltase). In this work we cloned an alpha glucosidase gene from the extremely halophilic archaea *Haloquadratum walsbyi*. The recombinant protein was purified and partially characterized.

## Literature Review

### I. Archaeal enzymes

Microorganisms are used as sources of enzymes for biotechnological applications because they can synthesize active products under conditions that can be controlled by humans and can multiply at extremely high rate (Gote, 2004). Extremophilic microorganisms present the advantages that have unusual and unique properties; that can be used in the development of novel biotechnological process (Schiraldi et al., 2002). These enzymes are important models, to learn how to design proteins with specific properties that are very useful in industrial applications (Vorgias et al., 2000).

Most of the industrial enzymes used to date have been derived from bacteria and fungi, and only a few archaeal enzymes have found their way to the market (Egorova et al., 2005). But, there are a large variety of enzymes that have been identified from different archaea, reflecting the metabolic diversity present in these organisms, which are greater than the diversity know in Bacteria and Eukarya (Siebers and Schonheit, 2005).

Enzymes derived from extremophiles can perform industrial processes, under harsh conditions, that detrimental to conventional proteins (Egorova et al., 2005). Archaeal thermophilic enzymes are the most studied in relation with haloenzymes. Some examples of the industrial uses of Archaeal thermophilic enzymes include: the use of glucoamylases from some thermoacidophilic archaea (*Thermoplama acidophilum*,

*Picrophilus torridus* and *Picrophilus oshimae*) to improve industrial glucose production. Thermostable cyclodextrin glycosytransferase is another enzyme discovered in Archaea that produces a series of cyclic dextrin from starch, amylase and other polysaccharides, that can be used as gelling, stabilizing agents in the food industry (Siebers and Schonheit, 2005). Others applications of the archaeal enzymes are the use of improving agents in food and paper industries by some xylanases obtained from *Pyroddictium abyssi* and *Thermococcus zilligi*i. In addition, heat stable enzymes are very important for the detergent industry because they can be used as additives for laundering.

In contrast, there is not much information available about purified and crystallized halophilic enzymes, because the high salt concentration at which these proteins have evolved plays an important role in the stabilization of their structure. It is well known that enzymes from halophilic organisms require a high salt environment for catalytic function and stability (Connaris et al., 1998). In adapting to the high osmotic stress prevalent in this harsh environment the cells maintain very high internal salt concentrations, up to 5M KCl, and their cellular components require high salt concentrations in order to be functional. This comprises a major disadvantage for haloenzymes purification because many of the common protein separation methods are ineffective at high salt concentration (Holmes et al., 1997). Also in contrast to the advantages of using *E.coli* as transformant cells such as rapid growth, high biomass level, and its well- known genetics, the expression of halophilic genes in its low ionic strength internal environment have proven very difficult (Connaris et al., 1998).

There have been many efforts to study different strategies and procedures in order to determine the best manner to produce desired halophilic enzymes in sufficient amounts for large scale use (Connaris et al., 1998). Some studies that deal with the use of halophilic archaeal enzymes are the following: the purification and biochemical characterization of an extracellular serine protease produced by *Natrialba madagii* (Jiménez et al., 2000); the characterization of a high stable α- amylase from the halophilic archaeon *Haloarcula hispanica* (Hutcheon et al., 2005); α-Amylase activity from the archaeon *Haloferax mediterrani* (Pérez et al., 2003), α- amylase gene cloning and molecular characterization from the moderate halophile *Halomonas meridiana* (Coronado et al., 2000); glucose dehydrogenase characterization from the halophilic archaeon *Haloferax mediterranei* (Bonete et al., 1996); characterization of an alcohol dehydrogenase from the haloalkaliphilic archaeon *Natronomonas pharaonis* (Cao et al., 2008) and the purification, sequencing and expression of the extremely halophilic β-galactosidase from *Haloferax alicantei* (Holmes et al., 1996; Holmes et al., 2000).

## II. Glycoside Hydrolases

Polysaccharides are the most abundant carbon source in biosphere and support heterotrophic growth in the three domains of life. Their utilization involves hydrolysis of them to generate monosaccharides (Verhees et al., 2003). Glycoside hydrolases and glycosyl transferases are important enzymes which are responsible for the hydrolysis and formation of glycosidic bonds. A glycosidic bond plays a central role in the

carbohydrates strcucture. (Seo Lee, 2004). Henrissat and coworkers, organized these enzymes into different families, based on amino acid sequence similarities and hydrophobic cluster analysis data. These classifications have the advantages that identify common structural domains, evolutionary connections and suggesting hydrolytic mechanisms for these enzymes (Sheridan et al., 2000). The classification started with 35 families in 1991, and has been constantly updated as the number of available sequences grows. Today it has grown to almost more than 100 families (Table 4).

Glycosyl hidrolases are classified with Enzyme Commision number as (EC 3.2.1.x). The first three digits indicate enzymes hydrolyzing 0-glycosyl linkages where the last number indicates the substrate and sometimes reflects the molecular mechanism. This unique classification is useful because avoid ambiguities and the proliferation of trivial names (Henrissat, 1991). The Carbohydrate Active Enzymes database (CAZy) provides a continuously updated list of the glycoside hydrolase families. Assigning a recently isolated glycosyl hydroalse to its corresponding family can be easily performed using databases and alignment software available on the internet (ENZYME, Protein Data Bank, Expasy, PROSITE).

Many families of glycosidases are polyspecific, containing enzymes of different substrate specificity with different EC numbers. On the other hand some glycoside hydrolases are multifunctional enzymes that contain catalytic domains that belong to different families (Davies and Henrissat, 1995). Also some families can be grouped into “clans” based on the three dimensional structural similarities of the proteins (Table 5).

Glycoside hydrolases are also referred to as glycosidases and sometimes also as glycosyl hydrolases.

**Table 4. Classification of some glycosidases's families of based on sequence similarity**

| Family | Enzyme and E.C. Number |
|---|---|
| **1** | β-glucosidase (3.2.1.21); β-galactosidase (3.2.1.23)<br>β-manosidase (3.2.1.25); β-glucorinase (3.2.1.31)<br>β-D-fucosidase (3.2.1.38); pholrin hydrolase (3.2.1.62)<br>exo-β-1,4-glucanase(3.2.1.74);6-phospho-β-galactosidase (3.2.1.85<br>6-phospho-β-glucosidase (3.2.1.86); lactase (3.2.1.108)<br>strictosidine β-glucosidase (3.2.1.105) |
| **5** | β-manossiadase (3.2.1.25); chitosanase (3.2.1.132);<br>cellulase (3.2.1.4); glucan 1,3-β-glucosidase(3.2.1.58)<br>licheninase (3.2.1.73); glucan endo-1,6-glucosidase (3.2.1.75)<br>mannanendo-β-1,4-mannosidase(3.2.1.78)<br>endo-β-1,4-xylanase (3.2.1.8); endo-β-1,6-galactanase (3.2.1.-)<br>cellulose β-1,4-cellobiosidase (3.2.1.91); xyloglucan-specific<br>endo-β-1,4 glucanase (3.2.1.151); β-1,3-mannanase(3.2.1.-) |
| **10** | endo-1,4-β-xylanase (3.2.1.8);endo-1,3-β-xylanase (3.2.1.38) |
| **15** | glucoamylase (3.2.1.3); glucodextranase (3.2.1.70)<br>α-trehalase (3.2.1.28) |
| **20** | β-hexosaminidase (3.2.1.52); lacto-N-biosidase (3.2.1..140)<br>β-1,6-N-acetylglucosamide(3.2.1.-);<br>β-,6-SO3-N- acetylglucosamide ( 3.2.1.-) |
| **25** | lysozyme (3.2.1.17) |
| **30** | glucosylceramidase (3.2.1.45); β-1,6-glucanase (3.2.1.75)<br>β-xylosidase (3.2.1.37); β-glucosidase (3.2.1.21) |
| **35** | β-galactosidase ( 3.2.1.23); exo-β-glucosaminidase (3.2.1.65) |

**Table 4. (Continued)**

| Family | Enzyme and E.C number |
|---|---|
| **42** | β-galactosidase (3.2.1.23) |
| **47** | α-mannosidase (3.2.1.113) |
| **52** | β-xylosidase (3.2.1.37) |
| **57** | α-amylase (3.2.1.1); α-galactosidase(3.2.1.22)<br>amylopullunase (3.2.1.41) |
| **62** | α-,L-arabinofuranoside (3.2.1.55) |
| **67** | α-glucorinase (3.2.1.139)<br>xylan α-1,2-glucorinase (3.2.1.131) |
| **72** | β-1,3-glucanosyltransglycosylase (2.4.1.-) |
| **77** | amylomaltase or 4-α-glucanotransferase (2.4.1.25) |
| **87** | mycodextranase ( 3.2.1.61); α-1,3-glucanase(3.2.1.59) |
| **82** | I-carrageenase (3.2.1.157) |
| **92** | mannosyl-oligosaccharide α-1,2mannosidase(3.2.1.113)<br>mannosyl oligosaccharide α-1,3-mannosidase (3.2.1.-)<br>mannosyl-oligosacharide α-1,6-manosidase(3.2.1.-);<br>α-,mannosidase (3.2.1.24); α-1,2-mannosidase (3.2.1-)<br>α-1,3-,mannosidase (3.2.1-); α-1,4-mannosidase (3.2.1.-) |
| **97** | α-glucosidase(3.2.1.20); α-,galactosidase (3.2.1.22) |
| **102** | peptisoglycan lytic transglycosylase (3.2.1.-) |
| **107** | sulfate fucan endo-1,4.fucanase (3.2.1.-) |
| **112** | galacto-N-biose phosphorylase (2.4.1.211)<br>D-galactosyl-1,4-L-rhamnose phosphorylase (2.4.1) |
| **115** | xylan α-1,2-glucoronosidase (3.2.1.131)<br>α-(4-O-methyl)-glucoronidase (3.2.1.-) |

**Table 5. Clans of Related GH Families**

| Clans | Related Families |
|---|---|
| GH-A | 1,2,5,10,17,26,30,35,39,42,50,51,53,5972,79,86,113 |
| GH-B | 7, 16 |
| GH-C | 11, 12 |
| GH-D | 27, 31, 36 |
| GH-E | 33,34,83,93 |
| GH-F | 43, 62 |
| GH-G | 37, 63 |
| GH-H | 13, 70, 77 |
| GH-I | 24, 46, 80 |
| GH-J | 32, 68 |
| GH-K | 18, 20, 85 |
| GH-L | 5, 65 |
| GH-M | 8, 48 |
| GH-N | 28, 49 |

Glycoside hydrolases (GH) are found in all domains of life. Some examples of their roles and functions are: breakdown and reassembly of edible carbohydrates, the processing of various oligosaccharide-containing proteins and lipids, the formation of cell walls and other barrier structures. They also function as cellular immuno-determinants (Balfour et al., 1993). In bacteria and archaea they are found both as intracellular and extracellular enzymes that are involved in nutrient acquisition. Glycosidases were grouped together based on the ability to hydrolyze similar substrates; for example β-galactosidases are the enzymes responsible for hydrolyzed lactose or its related chromogens o-nitrophenyl-β-D-galactopyranoside like (ONPG) and 5-bromo-4-chloro-3-indolyl--β-D-galactoside (XGal) (Sheridan et al., 2000).

The α-glucosidases are an important group of enzymes between the glycoside hydrolases, there have important roles in primary metabolism and in glycoconjugate biosynthesis and processing (Lovering et al., 2005).

**III. Glucosidases**

The enzymes responsible for hydrolyzing glucosidic bonds are named as glucosidases. There are two types of glucosidases: α-glucosidases and β-glucosidases depending on the configuration of the anomeric carbon atom of glucose in the substrate molecule on which they act.

**IV. Mechanism**

The mechanism of glycosidases has been the subject of many studies and the mode of action has been studied in great detail (Seo Lee, 2004). Glycosidases are classified into exo-glycosidases and endo-glycosidases based on the nature of the hydrolysis. The endo-glycosidases cleavages the glycosidic bond within the saccharide chain, in contrast the exo-glycosidases acts on the glycosidic bond present at the non reducing end of the sacharide chain (Gote, 2004). Hydrolysis of the glycosidic bond takes place via general acid catalysis that requires: a proton donor and a nucleophile/ base (Davies and Henrissat, 1995).

Glycosyl hydrolases operate via two general mechanisms leading to overall retention or inversion of the anomeric configuration (Fig. 13). An example of an

inverting enzyme is β-amylase, that hydrolyses α(1-4) bonds, but cuts off only maltose from the non reducing ends and forms a different anomeric configuration (β-D-glucose). On the other hand α-amylase is a retaining enzyme, because it hydrolases endo-α-(1-4) bonds in ploysacharides chains and produces linear α-(1-4) linked oligosaccharides (Gote, 2004). The retention of anomeric configuration works via a double displacement mechanism involving a covalent glycosyl-enzyme intermediate, but the inverting glycosidases works by a single chemical step involving the displacement of the aglycon-leaving group by a water nucleophile (Seo Lee, 2004)

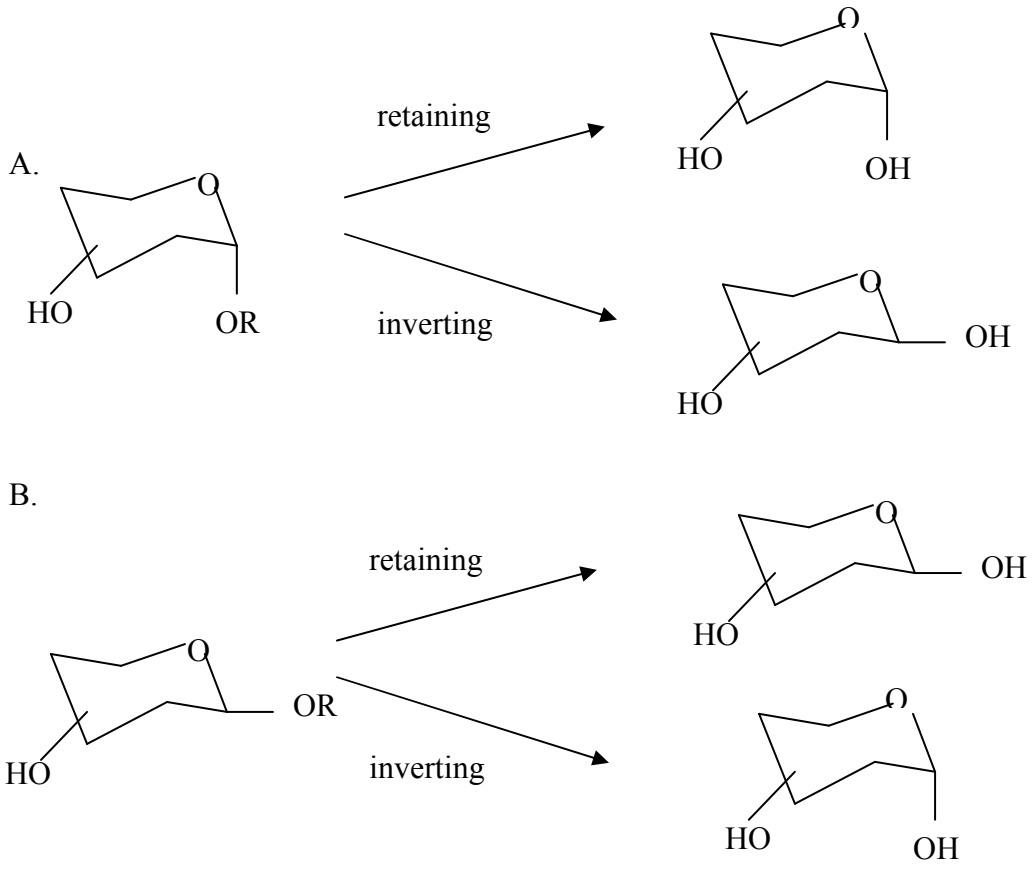


**Fig. 13. Diagram of the action mechanism of glycosidases: A. α- glycosidases; B. β-glycosidases (Seo Lee ; 2004).**

## V. Glycoside Hydrolase Family 31 (GH31)

GH 31 is a diverse family containing enzymes from all three domains of life and a range of different hydrolytic activities (Ernst et al., 2006). This family is one of the two major families of glycoside hydrolases that contain α-glucosidases (EC 3.2.1.20) enzymes. The other family is GH 13, the well-known α-amylase super family (Seo Lee, 2004). Family 13 contains a wide range of glucoside processing enzymes, including the α-amylases and cyclodextrin glucanotransferases. Both families share substrate specificity in large part, but amino acid sequence around the active site is very different to be grouped both families in a clan (Seo Lee, 2004). The GH 13 enzymes have been studied extensively in terms of three dimensional structures and mechanistics (Ernst et al., 2006). In the other hand, family 31 are represented from a range of organisms, including animals, plants and microorganisms but had received poor attention (Lovering et al., 2005).

In addition to α-glucosidases (EC 3.2.1.20), family 31 also contains the enzymes: α-xylosidases (EC3.2.1-), α-1,3 glucosidase (EC 3.2.1.84), sucrase-isomaltase (EC 3.2.1.48), the unique lytic enzyme α-1,4-glucan lyase (E.C.4.2.2.13) and isomaltosyltranferase (EC 2.4.1.). But the vast majority of the characterized enzymes of GH 31 are α-glucosidases. For all members of GH31, the catalytic function is the cleavage of a terminal carbohydrate moiety, while their substrate varies in size from small disaccharides to large storage polymers (Ernst et al., 2006). These enzymes play an important role in metabolism catabolism and in glycoprotein processing. Also these

enzymes can be found in all domains: Archaea, Bacteria and Eucarya. All of these enzymes worked by the retainment of the anomeric configuration mechanism. Diverse studies demonstrate that both the catalytic nucleophile/base and the catalytic proton donor corresponded to Asp, with the consensus sequence WIDMNE for plant, mammalian and fungal enzymes (Lovering et al., 2005; Ernst et al; 2006). For example in *Aspergillus niger* the residue that act as the catalytic nucleophile corresponds to Asp 224 (Lovering et al., 2005). And Asp 647 is the acid/base catalyst in *Schizosaccharomyces pombe* α-glucosidases (Lovering et al., 2005). However, bacterial proteins are know to have the sequence KTDFGE and are absolutely invariant. These differences between amino acids sequences of family 31 enzymes of bacteria and eukaryotes suggest that the bacterial proteins took a different path early in the evolution of GH family 31 (Lovering el at., 2005).

The region surrounding the catalytic nucleophile is highly conserved in the family and constitutes one of the two signature motifs of GH31 (PROSITE consensus motifs PS00129 and PS0007) (Ernst et al., 2006). Based on the proteins folding similarities GH 31 are grouped with the families GH 27 and GH36 in the clan D

**VI. Alpha glucosidases**

α-glucosidases (EC 3.2.1.20) are exoenzymes that hydrolyze the α-glucosidic linkages from the non-reducing end of oligossacharides and polysaccharides with the

release of α- glucose (Naested et al., 2006). These enzymes are distributed among five different glycoside hydrolases families (GH) in the sequence based classification system for carbohydrate –active enzymes; GH4, GH13, GH31, GH63, GH97 (Ernst et al., 2006). The α-glucosidases from higher organisms belong to the families GH31 and GH63, which have been less well characterized. The systematic name for α-glucosidases is α-D-glucoside glucohydrolases, but often are called: maltase, glucoinvertase, maltase-glucoamylase, α-glucopyranosidase, α-D-glucosidase, α-glucoside hydrolase and α-1,4 glucosidase. In the other hand α-glucosidases are named transglucosidases because some of them catalyze both hydrolysis and transglucosylation (Krasikov et al., 2001). The α-glucosidases that catalyze hydrolysis are classified by the Enzyme Commission with the EC number (3.2), both the glucosyltranferases are classified with the EC number (2.4).

These enzymes are conventionally classified depending on the type of preferred substrate. The first group (α-glucosidases I), prefers α-glucoside or sucrose rather than maltose. These groups hydrolyze aryl glucosides such as ρ-nitrophenol α-D-glucopyranose (pNPPG) faster than short malto-oligosaccharide (da Silva et al., 2009). α-Glucosidases II (maltases) catalyzes the hydrolysis only of maltooligosaccharides, but not of synthetic α-glucosides or sucrose. Finally, the third group (α-Glucosidases III) exhibits maltase activity; but their representatives can also hydrolyze α-glucans (like soluble starch and glycogen) (Krasikov et al., 2001; Marin et al., 2006; Wongchawalit et al., 2006).

These enzymes are widely distributed among microorganisms, plants and mammals. According to the classification of glycoside hydrolase family (GH), α-glucosidases I are members of GH 13 and type II and type III enzymes are members of GH 31 (Seo Lee., 2004; Wongchawalit et al., 2006).

## VII. Applications of α-glucosidases

α-Glucosidases are involved in the last step of starch degradation and are the second most important enzymes during the early stages of raw starch hydrolysis. α-glucosidases isolated from a variety of thermophiles or hyperthermophiles are potential candidates for processing starch into glucose syrup (Zhou et al., 2009). α-Glucosidases also catalyze transglucosyltation reactions that are exploited in biotechnology to produce food oligosaccharides or to conjugate sugar with biologically useful materials (Andreotti et al., 2006; da Silva et al., 2009; Zhou et al., 2009). In addition many papers report that inhibitors of α-glucosidases are potential therapeutics for the treatment of diseases such as viral diseases, cancer and diabetes (Hakamata et al., 2005).

## VIII. Occurrence and physiological role

α-Glucosidases are widely distributed in nature among microorganisms, plants, mammals and insects (Nishimoto et al., 2001).

**a) Plant**

α-Glucosidases are widely distributed in plants tissues like seeds, fruits, leaves and roots. Apparently the main function of these enzymes consists in the formation of glucose that is an energy source for the plant, by the hydrolysis of oligosaccharides produced through starch by α-amylases, β-amylases and dextrinases (Krasikov et al., 2000). Some reports suggest that in the absence of α-amylases, the degradation of natural starch granules in barley seeds and pea chloroplasts can be initiated by α-glucosidases (Yamasaki et al., 2007). Some reports show that α-glucosidases play an important role during the early stage of germinations in seeds (Yamasaki et al., 2005).

Plant α-glucosidases have been purified from chloroplast, pea, onion leaves, barley seeds, sugar beet, buckwheat, spinach, green pea, maize, and rice (Table 6). Like mammalian α-glucosidases, the plant enzymes can also be separated into groups of acidic and neutral glucosidases.

Multiple isoforms of α-glucosidases have been found in several plants and somes exhibit different substrates specificities (Taylor et al., 1998) Examples of substrates for hydrolysis include maltose, maltotriose, isomaltose, panose, kojibiose, soluble starch and native starch grain (Beers et al., 1990).

**Table 6. Occurrence of α-glucosidases activity among plants**

| Source | Reference |
|---|---|
| *Acremonium implicatum* | Yamasak*i* et al.,2005 |
| *Brassica oleracea* | Monroe at al.,1999 |
| *Hordeum vulgare* | Henson et al.,1990 |
| *Oryza sativa* | Nakai et al., 2006 |
| *Panicum miliacceum* | Yamasaki et al., 2004 |
| *Pisium sativum* | Beerset al., 1990 |
| *Scopelophila cataractae* | Yamasaki et al., 2007 |
| *Spinacia oleracea* | Sugimoto et al., 2003 |
| *Solanum tuberosum* | Taylor et al., 1998 |

**b) Animals**

Mammalian α-glucosidases can be separated into two main types: acidic α-glucosidases (pH optimum varies from 4 to 5) and neutral α-glucosidases (pH optimum varies from 6 to 7). Neutral α-glucosidases are widely distributed in mammalian tissues and body fluids, like blood serum and urine; both their biological role remains uncertain. At least three various neutral α-glucosidases were found in human urine; and pig blood serum was shown to contain high glucosidase activity (Krasikov et al., 2001). Acidic α-glucosidases from various animal tissues have been extensively studied. Acidic α-glucosidases are located in lysosomes of somatic cells and play an important role in glycogen metabolism. Pompe's disease in humans is a consequence of the deficiency of lysosomal α-glucosidase and cause death in infancy from cardio respiratory failure due to accumulation of glycogen in heart and skeletal muscle (Naested et al., 2006). Also α-glucosidases have been purified from insects like adult honeybees (Nishimoto et al., 2001)

**Table 7. Occurrence of α-glucosidases activity among mammals and insects**

| Source | Reference |
|---|---|
| *Apis cerana japonica* | *Wongchawalit* et al., 2006 |
| *Apis mellifera* | Nishimoto et al., 2001 |
| *Anopheles aquasalis* | Souza –Neto et al., 2007 |
| *Aplysia fasciata* | Andreotii et al., 2005 |
| *Coturnix japonica* | Kunita et al., 1997 |
| *Drosophila melanogaster* | Tanimura et al., 1979 |
| *Equs caballus* | Guidicelli et al., 1980 |
| *Homo sapiens* | Sim et al., 2008 |
| *Mus musculus* | Martini et al., 2007 |
| *Pichia pastoris* | Naested et al., 2005 |
| *Rana japonica* | Takesue et al., 1996 |
| *Sus scrofa* | Sorensen et al., 1982 |

**c) Microorganisms**

Various type of α-glucosidases with different substrate specificity have been found in microorganisms, where they present a very low affinity for polysaccharides and therefore attack starch at a very slow rate (Galichet et al., 1999). These enzymes were widely distributed among aerobic and anaerobic microorganisms, and could be found intracellular, extracellular or as a cell bound enzyme. These proteins have been extensively studied at the biochemical and molecular levels in a number of bacteria, but very little is know in archaea. α-Glucosidases have been described and purified from members of the archaea like *Sulfolobus shibatae, Pyrococcus furious, Pyrococcus woesi*, and *Thermococcus litoralis* but only one gene of archaea has been characterized in *S. solfataricus*. No alpha-glucosidases have been described so far for the extremely

halophilic archaea. Also these enzymes have been reported to be present in yeasts and fungi (Bun Li and Yu Chan., 1983).

**Table 8. Occurrence of α-glucosidases activity among microorganims**

| Source | Domain | Reference |
|---|---|---|
| *Bacillus subtilis* | Bacteria | Schonert et al., 1998 |
| *Enterobacter sakazakii* | Bacteria | Lehner et al., 2006 |
| *Escherichia coli* | Bacteria | Paul et al., 2008 |
| *Lactobacillus acidophilus* | Bacteria | Kwon et al., 1983 |
| *Lactobacillus brevis* | Bacteria | De Cort et al.,1994 |
| *Thermoanaerobacter ethanolicus* | Bacteria | Wang et al.,2009 |
| *Thermoanaerobacter tengcongensis* | Bacteria | Zhou et al., 2009 |
| *Thermococcus hydrothermalis* | Bacteria | Galichet et al., 1999 |
| *Thermotoga maritime* | Bacteria | Suresh et al.,2002 |
| *Thermus thermophilus* | Bacteria | Alarico et al., 2008 |
| *Aspergillus niveus* | Eukarya | da Silva et al.,2009 |
| *Aspergillus nidulans* | Eukarya | Kato et al.,2002 |
| *Aspergillus oryzaea* | Eukarya | Minetoki et al.,1995 |
| *Candida albicans* | Eukarya | Sturtevent et al.,1999 |
| *Mortierella alliacea* | Eukarya | Tanaka et al.,2002 |
| *Robina psuedoacacia* | Eukarya | Berthelot et al., 1999 |
| *Sccharomyces cerevisiae* | Eukarya | Dahanawansa et al.,2002 |
| *Saccharomycopsis fibuligera* | Eukarya | Reiser et al., 1995 |
| *Ferroplasma acidophilum* | Archaea(K.C) | Ferrer et al., 2005 |
| *Pyrococcus furiosus* | Archaea (K.C) | Constantino et al., 1990 |
| *Picrophilus torridus* | Archaea (K.C) | Angelov et al., 2006 |
| *Sulfolobus solfataricus* | Archaea (K.C) | Rolfsmeir et al.,1997 |
| *Thermococcus litoralis* | Archaea (K.C) | Xavier et al., 1999 |

*K.C=Kingdonm Crenarchaeota

Also genome sequencing projects have revealed a number of putative gene products from organisms that could be assigned to alpha glucosidases. The work presented in this chapter describes the cloning of a putative alpha glucosidase gene from

the extremely halophilic archaea *H. walsbyi* and the partial purification and characterization of the recombinant protein

## IX. *Haloquadratum walsbyi*

*Haloquadratum walsbyi* was described for first time in 1980 by Anthony E Walsby. It was detected by conventional microscopy in samples collected from a salt crust in the surface of a hypersaline pool on the Sinai Peninsula (Bolhuis et al., 2004). Such cells were commonly found as the dominant cell type in these and other waters including natural salt lakes and saltern crystallizer ponds, and were able to be characterized by 16S rRNA gene PCR and fluorescence *in situ* hybridization (FISH). But it was not until 2004 that the first isolates were obtained in pure culture by two independent groups: Bolhuis and coworkers from (Australia solar saltern) and Burns and coworkers from (Spanish solar slattern) (Burns et al., 2007). The isolates were preliminary named as "C23 and HBSQ001" (Bolhuis et al., 2004; Burns et al., 2004). Finally the genome of the strain HBSQ001 was published in the 2006 (Bolhuis et al., 2006).

This organism belongs to the group of halophilic archaea that is dominant in NaCl saturated thalassohaline waters around the world. Phylogenetically, the genus belongs to the family *Halobacteriaceae*. *Haloquadratum*'s cells are thin, square or rectangular sheets with sharp corners, many measuring 2-5 μm wide but no more than 0.2 μm thick

(Fig. 14). Also it contains gas vesicles and polyhydroxyalkanoate (PHA) storage granules (Walsby 2005). *H. walsbyi* is aerobic heterotroph, gram negative, non motile, pigmented and oxidase/catalase negative. Its optimal growth occurs under neutrophilic to alkaliphilic conditions, above 18% salinity and grows best on pyruvate as sole carbon source (Burns et al., 2007). Based on its 16S rDNA sequence it is a member of a novel genus within the family of *Halobacteriacea*. Their closest relative archaea was *Halogeometricum borinquense* with 91.2% of sequence similarity (Burns et al., 2007) (Fig. 15).

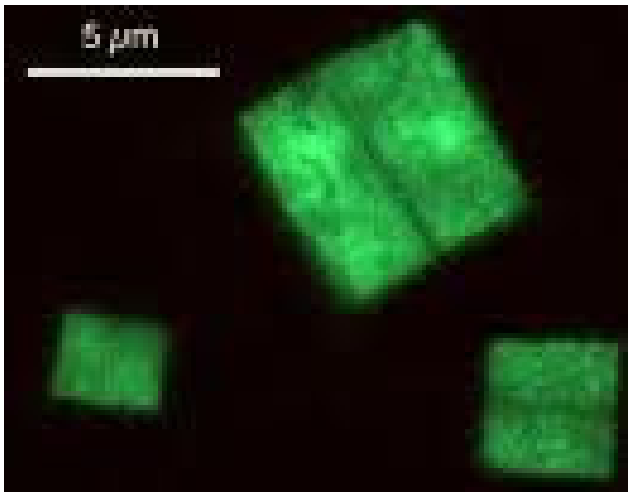


**Fig. 14**. **Acridine Orange stained pure culture of *H. Walsbyi* (viewed by fluorescence microscopy) (Bolhuis et al., 2004*).***

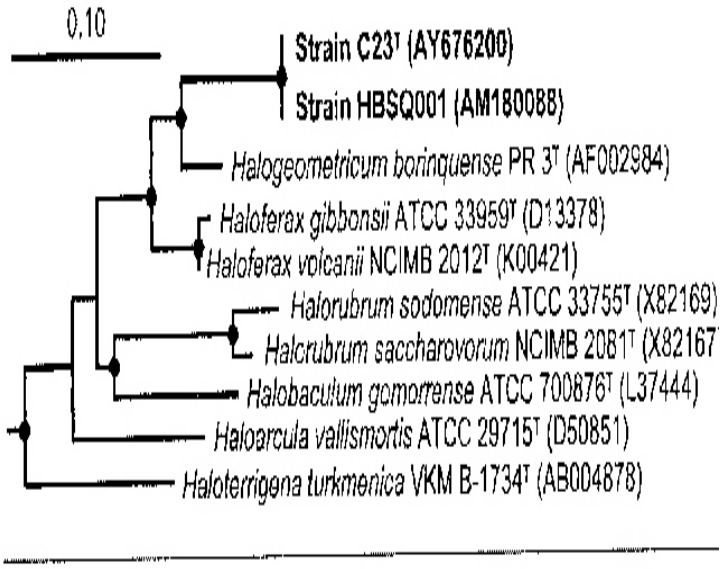


**Fig. 15. Phylogenetic tree reconstruction based on complete 16S rDNA gene sequences (Burns et al; 2007).**

## Molecular Biology Methods

### Strain

*Haloquadratum walsbyi* (Burns et al., 2007; JCM 12705$^T$, DSM No.16854. ) was kindly provided by Dr. Mike Dyall-Smith (Martinsried, Germany.).

### DNA extraction

DNA was extracted from cells using $H_20$dd as lysis solution. Two ml of the strain culture was centrifuged and the pellet was resuspended in 500 µl of distilled water. The extraction was performed at 70 $^0$C for 10 mins.

### Amplification of 16S rDNA

The gene encoding the 16 sRNA was amplified by PCR using the combination of forward primer ARCH 21-F (5'TTCCGGTTGATCCYGCCGGA) and with the reverse primer ARCH 958-R (5'YCCGGCGTTGAMTCCAATT). It was amplified by polymerase chain reaction (PCR), using TaKaRa La Taq DNA polymerase (5 U/µl). The reaction mixture was performed using a final volume of 50 µl and consisted of the following reactant concentrations: dd$H_2O$, GC Buffer II (2X), dNTPs (2.5 mM), primer forward (0.2µM), primer reverse (0.2µM), Takara La Taq (0.5 µl) and 1 µl of template (15 ng/ µl). The PCR reaction consisted of an initial denaturation cycle of 5 minutes at 95° C; 30 cycles with a denaturation period of 1 minute at 95°C, 1.5 minutes at 47°C , polymerization for 2 minutes at 72° C, and a final extension at 72° C for 5 minutes. The PCR product quality was verified on a 0.8% agarose gel stained with ethidium bromide.

**Isolation of the α-glucosidase gene in *H. walsbyi***

We used bioinformatics tools to analyze the genome of *Haloquadratum walsbyi* (http://www.genome.jp/kegg/pathway.html) for the presence of α-glucosidase gen sequences. Phylogenetically, this halophilic archaeon is closely related to *H. borinquense* (Burns et al., 2007) and it is possible that these organisms might share several genes with high homology. Previous studies made in our laboratoty demonstrated that *H. borinquense* has the ability to utilize maltose as a sole carbon source. The finding of a gene annotated as a *malA* in the genome of *H. waslbyi* was the starting point for the current study. A set of degenerate primers were designed for this ORF and a PCR reaction was performed using *H. walsbyi* genomic DNA. The putative maltase gene obtained from *H. walsbyi* using this approach was cloned and expressed in *E. coli Rosetta* as described below (Yi Cao et al., 2008). The recombinant protein was isolated and partially characterized as described below also (Yi Cao et al., 2008).

**PCR procedure for amplification**

The α- glucosidase gene (*malA*) was amplified by PCR using genomic DNA from *H. walsbyi*. It was amplified by polymerase chain reaction (PCR), using TaKaRa La Taq DNA polymerase. The sequences of forward and reverse synthetic primers which contained *Xho*I and *Nhe*I recognition sities were designed as 5'- CCA TAG CTA GCA TGT GGT TGG 3' and 5' -CGT CTC GAG ACC TCA GGA AGT ATT GG -3' respectively. The reaction mixture was performed using a final volume of 50 µl and

consisted of the following reactant concentrations: $ddH_2O$, GC Buffer II (2X), dNTPs (2.5 mM), primer forward (0.2µM), primer reverse (0.2µM), Takara La Taq (0.5 µl) and 1 µl of template (15 ng/ µl). The PCR reaction consisted of an initial denaturation cycle of 5 minutes at 95° C; 30 cycles with a denaturation period of 1 minute at 95°C, 1.5 minutes at 47°C , polymerization for 2 minutes at 72° C, and a final extension at 72° C for 5 minutes. The PCR product quality was verified on a 0.8% agarose gel stained with ethidium bromide. PCR amplicons were purified using the SV Gel and PCR Clean-UP System (Promega, Madison, WI, USA) according to the manufacturer instructions and the product concentration was determined using a nanodrop instrument (ND 1000, Fisher Inc)

**Construction of the expression vector**

The PCR product obtained from *H. walsbyi* was digested with the restriction enzymes *Xho*I and *Nhe*I (New England Biolabs Inc). The expression vector was constructed using the plasmid pET 28b(+) (Novagen, Germany), which also was digested with *Nhe*I and *Xho*I (New England Biolabs Inc.). Ligation of the digested PCR amplicon and the plasmid was performed using T4 DNA Ligase (Promega Inc.) in a molar ratio insert / vector of 3:1 respectively. The ligation reaction was performed for 4 hr at room temperature. The resulting construct was designated pET- malA.

**Transformation**

The recombinant plasmid was transformed into *E.coli Rosetta* ™ 2(DE3) Singles Competent Cells (Novagen Germany) by heat shock, according to the manufacturer's instructions.

**Colony PCR**

The colony PCR technique (Gussow and Clackson, 1989) was selected to determine the present of the insert in the vector. The reaction mixture consisted: of dd$H_2O$, GC Buffer II (2X), dNTPs (2.5 mM), primer forward (0.2µM), primer reverse (0.2µM), Takara La Taq (0.5 µl) and 2 µl of template. Samples and controls were fractionated through gel electrophoresis in agarose 0.8% and stained with ethidium bromide.

**Miniprep (Lysis Alkaline)**

Plasmid extraction and purification were performed using the Quiagen Kit, according to the manufacturer's instructions.

**Protein expression and purification**

The transformed *E. coli* cells were grown in 3L of Luria Bertani Broth (containing 34 µg/ml chloramphenicol and 30 µg/ml kanamycin) at 37 $^0$ C. After the turbidity at 600 nm reached 0.6-0.8, 1mM isopropyl-D-thiogalactopyranoside (IPTG) was added to the culture. Following an induction period of 3h, the cells were harvested by centrifugation (4,000 rpm x 20 min. at 4 $^0$C). This necessitated either reactivation or refolding of the recombinant halophilic enzyme. The inclusion bodies were purified by denaturing the enzyme in 6M guanidine HCl or in urea, and slowly renaturing by dialysis versus dilute buffer. Previous studies have revealed that halophilic enzymes are usually unstable in

chaotropic salts such as urea or guanidine hydrochloride (Hutcheon et al., 2005). After these results we decided to purify the little soluble fractions of protein that could be in the supernatant culture following the instructions provided by Quiaexpresionits handbook guide.

The pellet was resuspended in cold sodium phosphate buffer (50mM $Na_2HPO_4$, 10mM imidazole, 3M NaCl; pH 8.0) and incubated on ice with 1mg/ml of lysozyme for 30 min. Finally the cells were broken by sonication on ice and the supernatant obtained by centrifugation (4,000 rpm x 20 min at $4^0$C) was the crude enzyme extract.

The enzyme was purified by Ni-NTA agarose chromatography at room temperature. The crude enzyme extract was loaded onto a chromatography column filled with Ni-NTA agarose (Qiagen, Germany) and washed four times with sodium phosphate buffer with a 20mM imidazole concentration, to remove unspecifically bound proteins. Finally the histidine tagged protein was eluted in sodium phosphate buffer containing 250mM imidazole concentration.

**Electrophoresis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% acrylamide gels and the proteins were visualized by staining with Coomassie G250 stain (Bio Rad). Precision Plus Protein Kaleidoscope marker (Bio Rad) was used as the protein molecular mass standard.

**Protein assay**

Protein concentration was determined by the Pierce BCA Protein Assay kit, using bovine serum albumin (BSA) as a standard.

**Enzyme assay**

The α- glycosidase activity was determined by measuring the formation of p-nitrophenol (pNP) from the hydrolysis of p-nitrophenyl α-D glucopyranoside (PNPG, the typical substrate for α- glycosidase) at 40 $^{0}$C. It was monitored using a spectrophotometer at 420 nm. PNPG is an artificial substrate in which a chromophore para –(or 4-) nitrophenol is present. When the substrate is hydrolysed, p-nitrophenol is formed. At alkaline pH the phenolic proton dissociates giving a phenolate anion with an intense yellow colour that can be easily measured in a spectrophotometer. The reaction mixture consisted of the substrate prepared at 10mM, and it was added in a reaction buffer consisting of 50mM Tris hydrochloride, 3M NaCl (pH 7.2). Reactions were initiated by the addition of the enzyme to the substrate solution and terminated by the addition of 500 µl 1M sodium carbonate. A unit of either α-glucosidase activity is defined as the amount of enzyme required to liberate 1 µmol of ρ-nitrophenol per min/ mg of total protein.

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

The optimum temperature was determined under standard assay conditions by incubating the reaction mixture at temperatures ranging from 20 to 70$^{0}$C; pH 7 and 15% NaCl. For determining the optimum pH for enzyme activity, a standard assay mixture in

100mM of the following buffers was used: (citric acid [pH 3-6] and Tris HCl [pH 7-9]), at 40 $^{0}$C and15 % NaCl. The effect of salt concentration on enzyme activity was tested by measuring the activity in buffer containing different NaCl concentrations (5, 10, 15, 20, 25, 30, 35 %w/v), at 40 $^{0}$C and pH 6.

**Bioinformatic tools**

To obtain information about the location of the gene in the chromosome and predicted biochemical characterization of the protein was necessary the use of different data bases: Pfam (http://pfam.sanger.ac.uk/family?entry=PF01055), Expasy Proteomics Server (http://expasy.org/), Inter Pro Scan (http://www.ebi.ac.uk/Tools/InterProScan/), NCBI Conserverd Domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), "DAS–Transmenbranal Predictor Server(http://www.sbc.su.se/~miklos/DAS/tmdas.cgi), TMpred(http://www.ch.embnet.org/software/TMPRED_form.html), and HMMTOP (http://www.enzim.hu/hmmtop/).

**Results**

In silico analysis of the *Haloquadratum walsbyi* genome at the Kegg database, (http://www.genome.jp/kegg/pathway.html), revealed an open reading frame potentially coding for a putative alpha glucosidase (Fig. 16). With the use of different databases (Pfam database, Expasy Proteomics Server, PROSITE, Inter Pro Scan, NCBI Conserverd Domains, "DAS –Transmenbranal Predictor Server, TMpred, HMMTOP) and primary annotation, we determined the position of the gene in the chromosome of *H. walsbyi*, the predicted molecular weight of the coded protein, and its biochemical characterization, which includes the presence of two putative transmembrane helices (Fig. 18). The coordinates of the gene in the chromosome are between 1108309-1105961 and the ORF codifies for a polypeptide of 782 aa (Fig. 17).

The three strains of *Haloquadratum walsbyi* that were kindly provided by Dr. Mike Dyall-Smith (Martinsried, Germany.) were used to extract their genomic DNA (Fig. 19). To confirm the nature of the strains provided, the genomic DNA extraction was used as template for 16s rDNA amplification by PCR (Fig. 20).

```
>hwa:HQ1911A malA; alpha-glucosidase (N)
atgtggttggagctcacagaggtgtgtgggactgattttgaacgaccgtttgaatttgac
tgtgcggctaagccgctaaataacgaacatccgactcccagacatactattccaatttca
gtgcagttcctcactgagaaagccgtcagactggtccttcgaccaaatccagaggtagtt
ggagtggactcttctccattaggtctgccgtatgatgagtattctcgtgatacaaacgtt
gatataaactcagaagatgaaactgtcaagctcgatactgagggcatttcggcttcagtc
gctaccgacaccgcttcgtttactctgagttacgataacaaacggatactcgacactaat
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attgtcgacaactacccgcttgaagtggtacaaacgggcttctccaccgcgcttgaccct
gcggaatcgatattcggactgggagaacagttcactacctttgaaaagagcggtagtaga
```

```
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ccgtttttctatctgatcgtggctacggcgttcttgttgagacggcgtgtgatgttaca
ttcgacttcgggagcaatacgccggatgcgacggcgattaatgttgattcgtctgttctc
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gatgaatcgtggcagataatgacagttacgacatcagacagtctaccgcctggaactgtc
atcgtggaagccgccgctaacccacccaacagggttgttgttgatgacacggaacttgat
tcagacgagatacagtatgatgctaacgagaggatactcaccttcgacatcaactccaat
acttcctga
```

**Fig. 16. DNA sequence corresponding to the putative gene *malA* (ORF: HQ1911A) in the *Haloquadratum walsbyi* genome (**(http://www.genome.jp/kegg/pathway.html**). The ORF was identified as an alpha glucosidase gene.**

```
>hwa:HQ1911A malA; alpha-glucosidase (A)
MWLELTEVCGTDFERPFEFDCAAKPLNNEHPTPRHTIPISVQFLTEKAVRLVLRPNPEVV
GVDSSPLGLPYDEYSRDTNVDINSEDETVKLDTEGISASVATDTASFTLSYDNKRILDTN
IDVTNNRGELSVPTIGYKENIVDNYPLEVVQTGFSTALDPAESIFGLGEQFTTFEKSGSR
VEASVSQAHGTNSNDTYAPVPFFLSDRGYGVLVETACDVTFDFGSNTPDATAINVDSSVL
SIVVFAGESLKDIISSYTALTGRAPELPEWTYGIWMSRNSYESQTQVRDIASEIRERSMP
CDVLHVDPGWMDMEAPEMAFDAEEFPSPEKMKANLAEAGFRLSVWEYPYINTGTDLFRAA
EQNDYLVTDHEGRSYILRRPSYSATRAGIIDFSNPEAISWWQEIHHELIESGIDVFKTDF
GEYLPPQTTTADRRTGMGGKNIYSVAYQRAVAGAFEEFDKPPVLWSRSAWVGAQQYPIHW
GGDTRSTFKGFRESVRGGLSLLISGFQFWSCDIGGYKPKPSETLYIRWAQWALLSLSHPR
FHGKTPREPWMFGDRAAKIIIEFAKLRYRLLPYYLSYGCEAIATGVAIMRPMALEFEDYQ
QVSASATQHMIGEEFLVAPVLSVDGRVKVDLPPGEWVDYWSGEYHVGPQRQRREPNLDEL
PFFVRAESIIPEDPRVRMHADGPPAELDYRVYPACEGKTTTQFTVRHPEVKNPDTIEVEI
DESWQIMTVTTSDSLPPGTVIVEAAANPPNRVVVDDTELDSDEIQYDANERILTFDINSN
TS
```

**Fig. 17. Amino acid sequence corresponding to the ORF:HQ1911A. The predicted weight of the protein based on this sequence is 87 kDa.**

```
Protein: mal A
Length:  782
N-terminus: OUT
Number of transmembrane helices: 2
Transmembrane helices: 497-516 570-589

The best path:

     seq  MWLELTEVCG TDFERPFEFD CAAKPLNNEH PTPRHTIPIS VQFLTEKAVR    50
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  LVLRPNPEVV GVDSSPLGLP YDEYSRDTNV DINSEDETVK LDTEGISASV   100
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  ATDTASFTLS YDNKRILDTN IDVTNNRGEL SVPTIGYKEN IVDNYPLEVV   150
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  QTGFSTALDP AESIFGLGEQ FTTFEKSGSR VEASVSQAHG TNSNDTYAPV   200
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  PFFLSDRGYG VLVETACDVT FDFGSNTPDA TAINVDSSVL SIVVFAGESL   250
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  KDIISSYTAL TGRAPELPEW TYGIWMSRNS YESQTQVRDI ASEIRERSMP   300
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  CDVLHVDPGW MDMEAPEMAF DAEEFPSPEK MKANLAEAGF RLSVWEYPYI   350
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  NTGTDLFRAA EQNDYLVTDH EGRSYILRRP SYSATRAGII DFSNPEAISW   400
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  WQEIHHELIE SGIDVFKTDF GEYLPPQTTT ADRRTGMGGK NIYSVAYQRA   450
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  VAGAFEEFDK PPVLWSRSAW VGAQQYPIHW GGDTRSTFKG FRESVRGGLS   500
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO Oooooooooo ooooooHHHH

     seq  LLISGFQFWS CDIGGYKPKP SETLYIRWAQ WALLSLSHPR FHGKTPREPW   550
     pred HHHHHHHHHH HHHHHHiiii iiiiiiiiii iIIIIIIIII IIIIIIIIII

     seq  MFGDRAAKII IEFAKLRYRL LPYYLSYGCE AIATGVAIMR PMALEFEDYQ   600
     pred IIIIiiiiii iiiiiiiiiH HHHHHHHHHH HHHHHHHHHo oooooooooo

     seq  QVSASATQHM IGEEFLVAPV LSVDGRVKVD LPPGEWVDYW SGEYHVGPQR   650
     pred ooooOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  QRREPNLDEL PFFVRAESII PEDPRVRMHA DGPPAELDYR VYPACEGKTT   700
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  TQFTVRHPEV KNPDTIEVEI DESWQIMTVT TSDSLPPGTV IVEAAANPPN   750
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO

     seq  RVVVDDTELD SDEIQYDANE RILTFDINSN TS  782
     pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OO
```

**Fig. 18. Transmenbranal helices predicted by HMMTOP database**

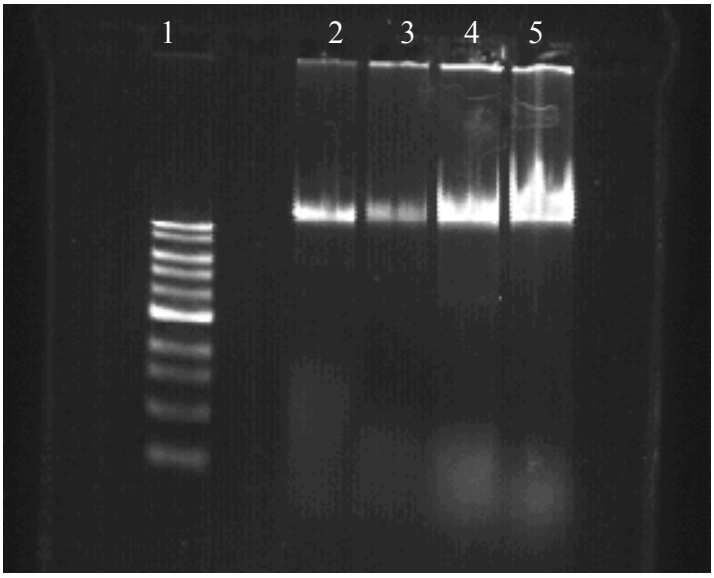


**Fig. 19. Genomic DNA extraction for *H. walsbyi* strains Lane 1: KB ( 100 bp DNA ladder;Biolabs); Lane 2 HC23 DNA extraction; Lane 3 HBSQ001 DNA extraction; Lane 4 -5C23 DNA extraction.**

**937 bp~**

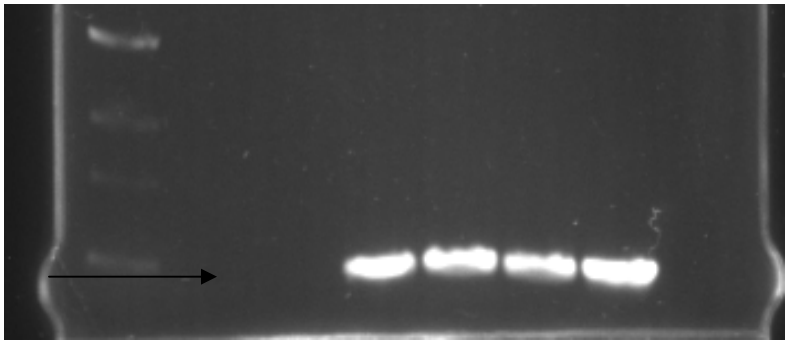

**Fig. 20. Amplification of the 16s sDNA using forward primer ARCH 21-F and reverse primer ARCH 958-R. PCR amplicons were of approximately 937bp long.**

Primers were designed using the sequence of the putative alpha glucosidase ORF gene from *H. walsby* strain C23, and cloned into the protein expression vector pET 28b(+)(Novagen, Germany) (Fig. 21). Primer forward were designed starting 11 bp

upstream and primer reverse 12 bp downstream from the open reading frame. The competent cells used were *E.coli Rosetta* ™ 2(DE3) Singles Competent Cells (Novagen Germany). Plasmid extraction and colony PCR were made in order to corroborate the presence of the correct insert in the transformants (data not shown).

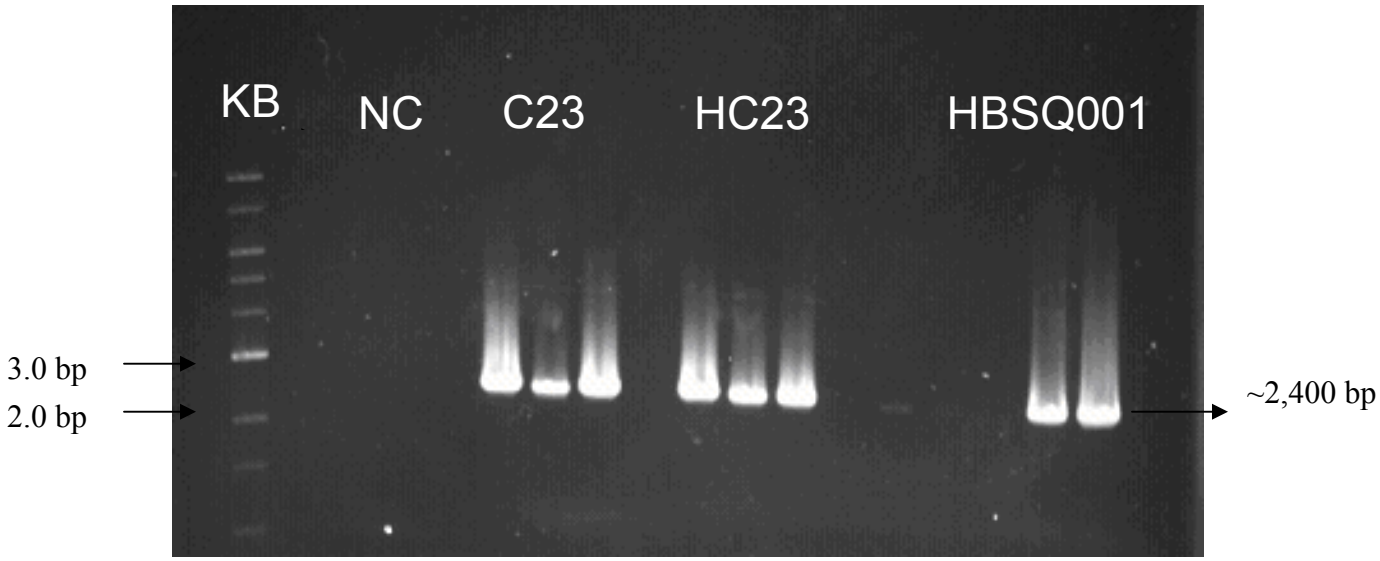


**Fig. 21. Amplification of the putative alpha glucoosidase gene from some strains of *H. walsbyi*: C23, HC23, HBSQ001. (See materials and methods). It was to perform optimization of the PCR parameters in order to achieve optimal amplifications. PCR product had a size of nearly 2,400 bp.**

In order to generate large quantities of the alpha glucosidase enzyme, *E.coli Rosetta* carrying the plasmid pET-malA was grown in Luria-Bertani medium, and the production of the recombinant enzyme was induced with IPTG. The recombinant alpha glucosidase enzyme was partially purified by Ni-NTA chromatography methods and the expected fraction was detected in 10% SDS-PAGE (Fig. 22). After staining with Coomassie dye, a protein band with an apparent molecular mass of approximately 87 kDa

was detected in the lanes of the protein elutions purified with Ni-NTA agarose chromatography methods. As expected, this protein band was absent in the lane corresponding to proteins migration of *E.coli Rosetta*. The purified enzyme was stable for various weeks at 20 $^{0}$C.

To demonstrate the expression of the alpha glucosidase recombinant enzyme, we calculated the specific activity for the following extracts: *E.coli Rosetta* grown cells, Transformant cells (TC23) grown without IPTG, Transformant cells (TC23) grown with IPTG and eluted fractions that showed alpha glucosidase activity (Table 9 and Fig. 23). The enzymatic assays were made in duplicate and the substrate used was p-nitrophenyl α-D glucopyranoside (PNPG), the typical substrate for α- glycosidase. The purified protein showed a specific activity of 163 ($\mu$moles PNPG/min/mg) versus 0.350 (μmoles PNPG/min/mg) of *E.coli* Rosetta extract cells.

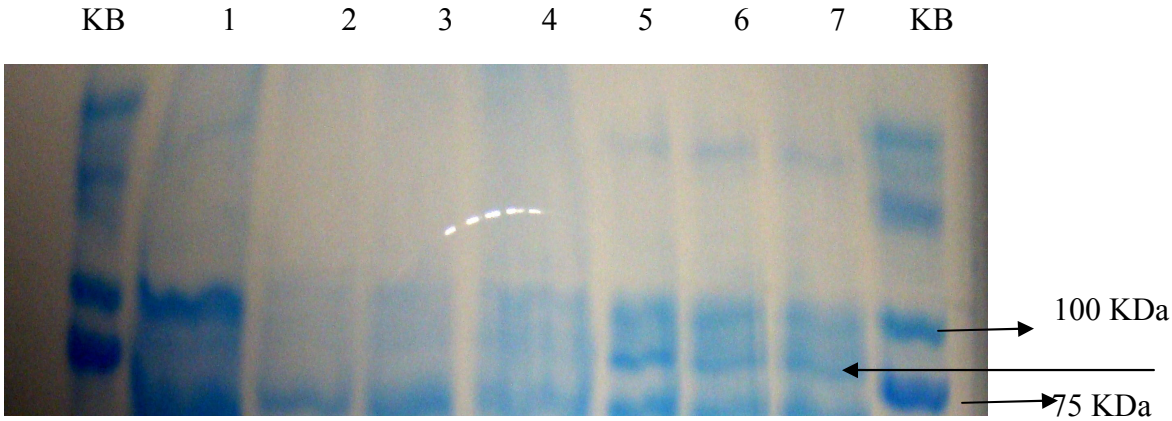


**Fig. 22. SDS-PAGE with the purification of the *Haloquadratum waslbyi* α-glucosidase using Ni-NTA chromatography methods. Precision Plus Protein Kaleidoscope market (Bio Rad) was used as the protein molecular mass standard. (see Materials and methods). Lane 1: Proteins bands from *E. coli Rosetta* grown cell, lane 2: TC23 cells grown without IPTG , lane3: TC23 cells with IPTG grown, lane 4: Wash purification elution, lane 5-7: eluted fractions that showed alpha glucosidase activity. The arrow indicates the alpha glucosidase protein band, which approximately weights 87 KDa.**

**Table 9. Expression of the recombinant enzyme in the eluted fractions and in the following cells extracts: *E.coli Rosetta* cells, Tranformant cells (TC23) grown without IPTG, Tranformant cells grown with IPTG.**

| Cells Extracts | Specific Activity (µmoles PNP/ min/mg) |
|---|---|
| E. coli Rosetta | 0.350 |
| TC23 withouth IPTG | 2.370 |
| TC23 with IPTG | 13.310 |
| Protein elution Fraction | 163.230 |

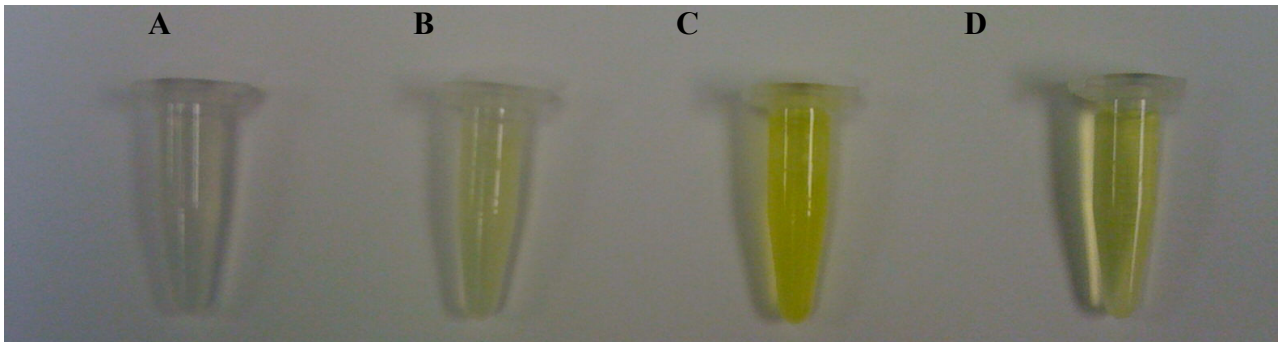


**Fig. 23. Colorimetric enzymatic assays reactions of the following extracts: A. *E.coli Rossetta*, B. TC23 with out IPTG, C. TC23 with IPTG and D. Protein elution fraction. (See materials and methods)**

The effects of temperature (Figs. 24 and 25), salt concentration (Figs. 26 and 27) and pH (Figs. 28 and 29) were tested. The enzymatic activity of the enzyme was measured at different temperatures ranging from 20 $^0$C to 70 $^0$C. In addition it was measured at different salt concentration (0% - 35% (w/v) NaCl) and the pH optimum of the enzyme was determined with buffers with pH values ranging from 3 to 9. The experiments were performed in duplicate. The optimum temperature of the enzyme was 40 $^0$C, with more than 50% activity remaining at 50 $^0$C and 38% at 30$^0$C. At higher or lower temperatures the activity dropped quickly with only near to 10% and 6% of activity remaining at 20 $^0$C and 70 $^0$C respectively. The recombinant enzyme displayed optimal activity at a pH of 6.0. Activity decreased quickly in more acidic or alkaline conditions. More than 70% of the activity was lost at a lower or higher pH than 6.0. The most characteristic of halophilic proteins is the ability to function under very saline conditions. The recombinant enzyme is indeed extremely halophilic, as it functions at salt conditions close to 3M NaCl (15 % NaCl). In contrast to other halophilic proteins, the enzyme did not lose all of its activity at low salt concentrations; in the absence of NaCl the enzyme still had more than 10% of its activity.

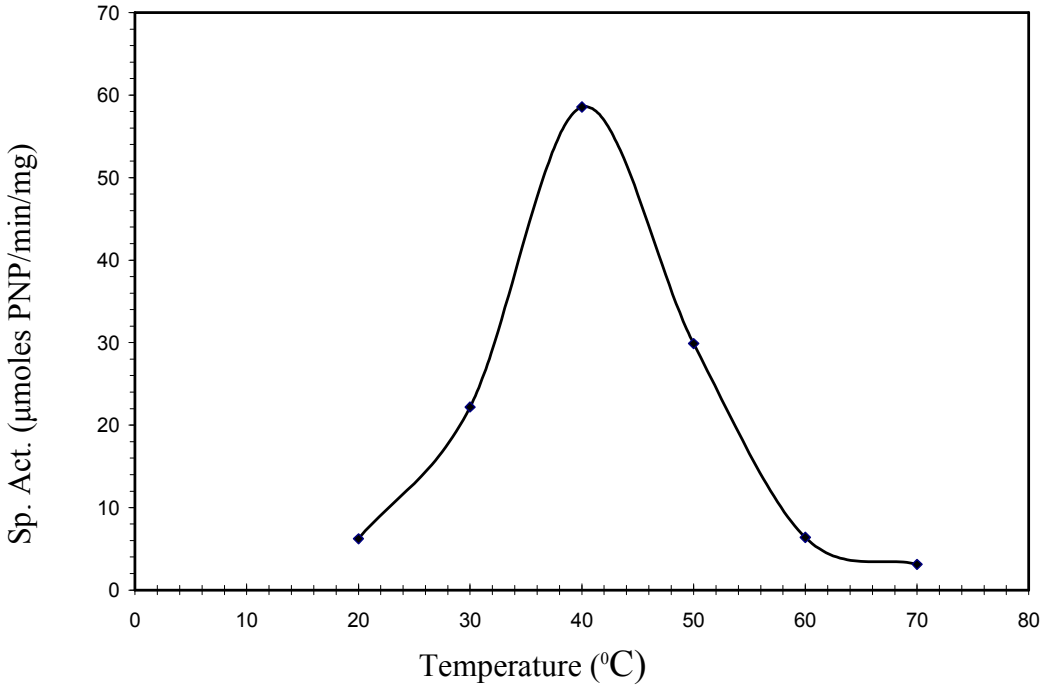


**Fig. 24. Effect of temperature on *Haloquadratum walsbyi* recombinant enzyme. Specific activity of the purified enzyme was measured at different temperatures (20 $^0$C -70 $^0$C). Experimental procedures are described under Materials and Metods.**

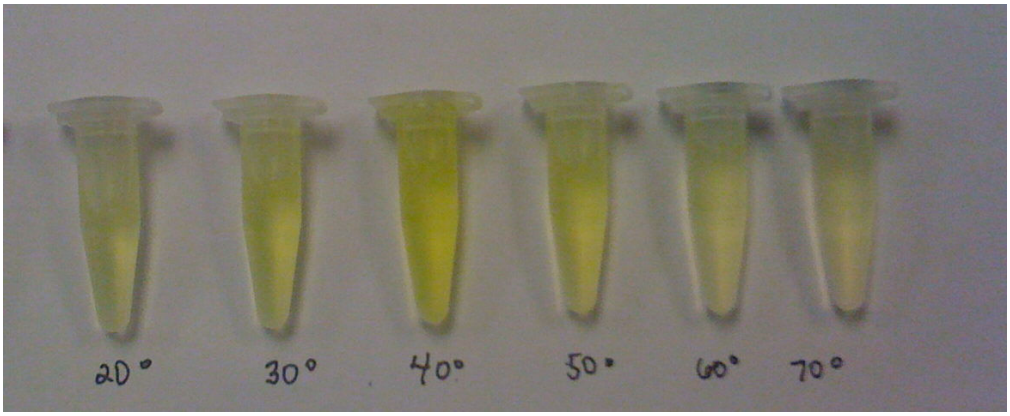


**Fig. 25. Colorimetric enzymatic assays results of the purified protein at different temperatures ranging from 20 $^0$C to 70 $^0$C.**

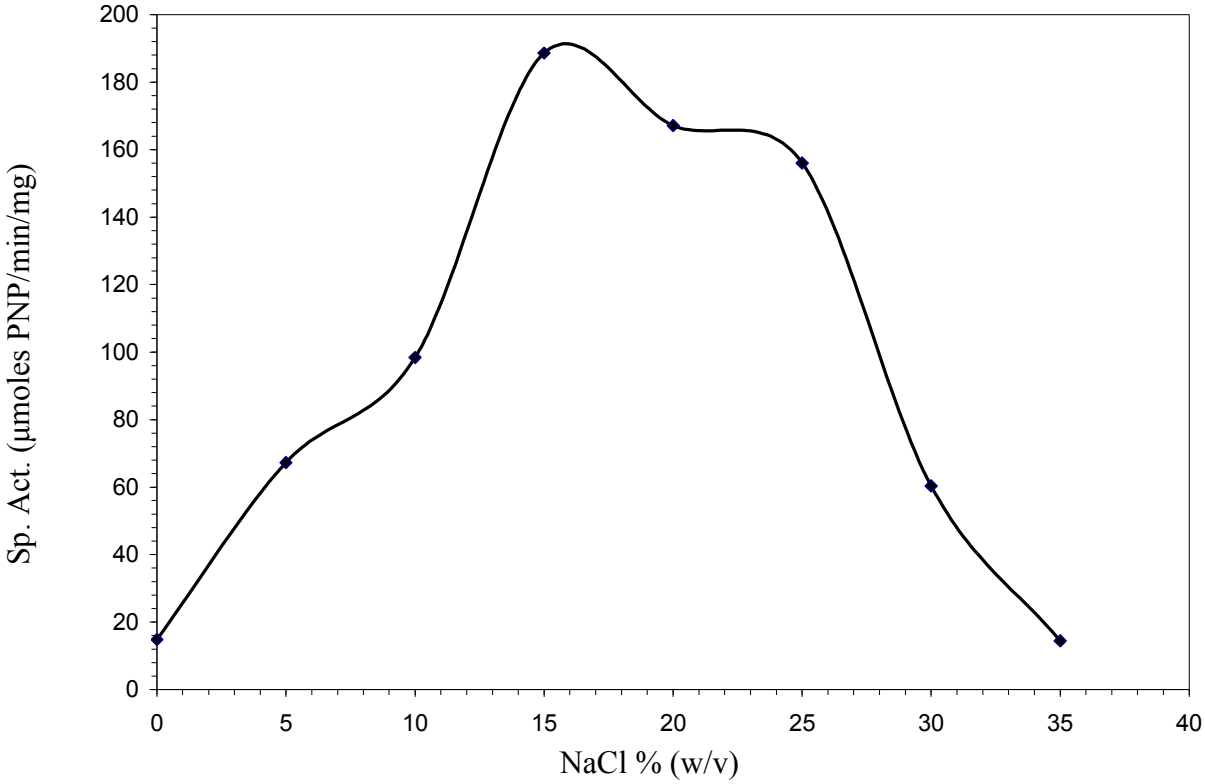


**Fig.26. Effect of salt concentration on *Haloquadratum walsbyi* recombinant enzyme. Specific activity of the purified enzyme was measured at salt (NaCL) percent (0%-35 $^{0}$C). Experimental procedures are described under Materials and Methods.**

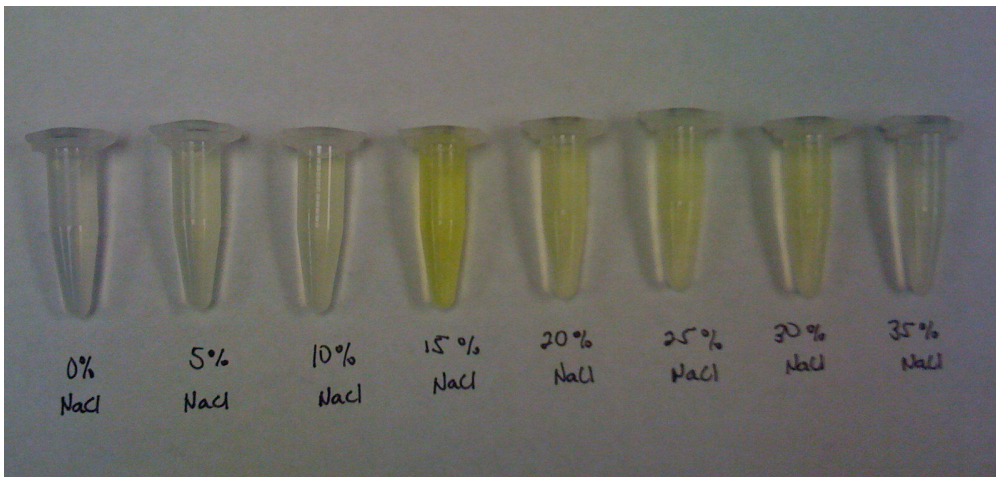


**Fig. 27. Colorimetric enzymatic assays results of the purified protein at different NaCl percents.**

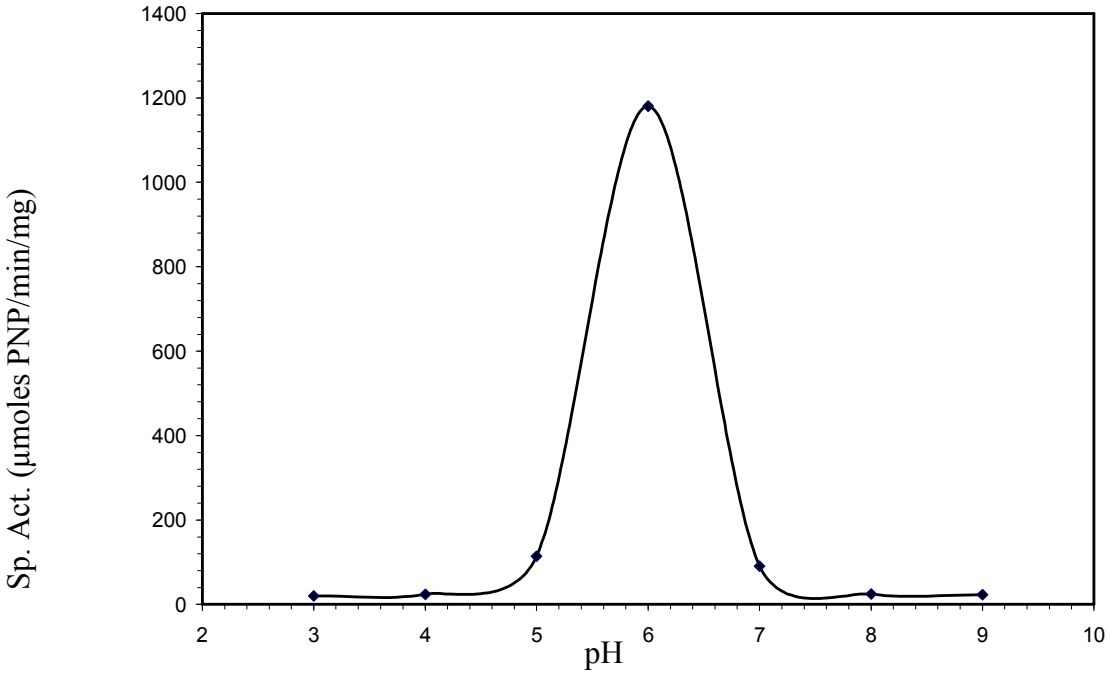


**Fig. 28. Effect of pH on *Haloquadratum walsbyi* recombinant enzyme. Specific activity of the purified enzyme was measured at different pH ranging from 3 to 9 Experimental procedures are described under Materials and Methods.**

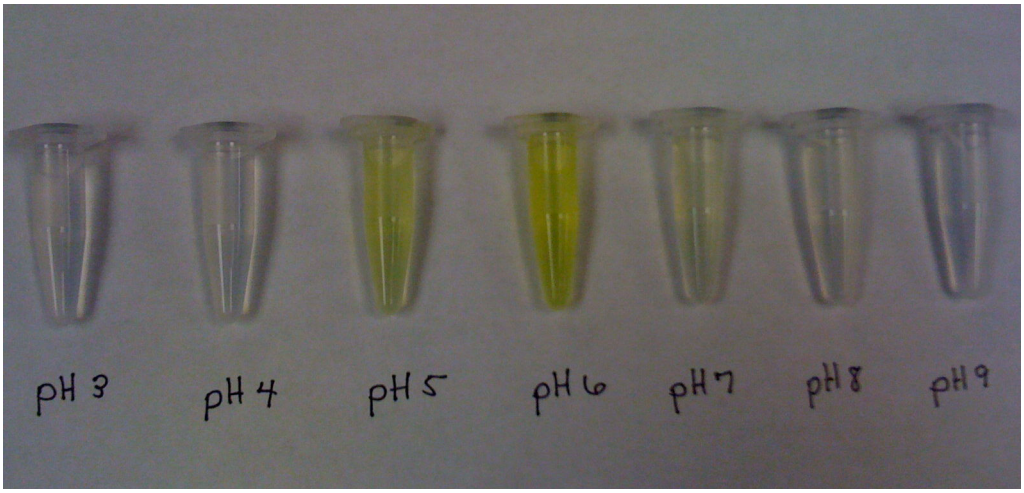


**Fig. 29. Colorimetric enzymatic assays results of the purified protein at different pH ranging from 3 to 9.**

**Discussion**

Alpha glucosidases are part of the carbohydrate metabolism machinery that allows the archaea to utilize carbohydrates, such as maltose, as the sole carbon and energy source. Alpha-glucosidases have been purified from representatives of all three phylogenetic domains. Many α-glucosidase genes from eukaryotic and bacterial organisms have been cloned and characterized, and the majority of them have been from mesophilic organisms. The previously reported archaeal α-glucosidase enzymes are from *Sulfolobus solfataricus*, *Pyrococcus furious*, *Thermococcus litoralis*, and *Ferroplasma acidophilus*, all of these members of the kingdom *Crenarchaeota*. Although such enzymes also occur among the archaea, none of their corresponding genes have yet been characterized from halophilc members of the kingdom *Euryarchaeota*. There are no reports in the literature describing an extremely halophilic alpha glucosidase (maltase). In addition, there is not much information available about purified and crystallized halophilic enzyme, due to the high salt role in stabilizing these proteins which makes them difficult to purify.

Originally, the objective was work with *Halogeometricum borinquense* (Chapter 1) because we found that this organism use maltose as a carbon source and has detectable levels of maltase activity. In addition, the experiments performed in Chapter 1 may indicate the presence of a putative carbon catabolic repression system (CCR) in this organism. However, the isolation and characterization of the *H. borinquense*'s maltase gene proved to be an extensive procedure requiring reverse genetics experiments due to

the lack of access to the genome at the moment. Therefore, we decided to work with the halophilic archaea *Haloquadratum walsby.* Based on its 16S rDNA sequence, *H. walsbyi* is a member of the family of *Halobacteriace*a, and it is phylogenetically related to *H. borinquense* with 91.2% of sequence similarity. Also the genome of *H. walsbyi* was sequenced and available in the Kegg database (http://www.genome.jp/kegg/pathway.html). The predicted amino acid sequence of ORF HQ1911A from the genome of *H. walsbyi* codifies for a putative alpha glucosidase enzyme. All non redundant data bases were screened for entries showing similarity to this ORF with the BLASTP program. The predicted gene product deduced from the 2,349-bp DNA sequence exhibits high similarity to enzymes of family 31 of glycosyl hydrolases.

In this work we expressed and partially characterized a recombinant alpha-glucosidase in *E. coli Rosetta* competent cells from the extremely halophilic archaeon *H. walsbyi*. This represents the first report of the expression of a recombinant α-glucosidase enzyme from a halophilic member of the *Euryarchaeota* archaeal kingdom. Several attempts made by myself and other laboratory members to obtain partial amino acid sequence from this recombinant protein were unsuccessful, but through enzymatic assays we can demonstrate the expression of the recombinant enzyme. The colorimetric maltose analog PNPG was used as a substrate to monitor maltase activity during the purification procedure. The function of the *H. walsbyi* α-glucosidase in vivo remains unknown, so does the normal substrate for the enzyme. At this moment we do not know if this enzyme

have activities other than maltase. To obtain more information about this, it could be necessary to study the substrate preference of the *H.walsbyi* alpha glucosidase enzyme.

Ni-NTA agarose chromatography method was used to purify the enzyme (little soluble fractions of protein that could be in the supernatant culture) at room temperature. Previously, some experiments were performed with the objective of knowing the solubility state of the protein. The absence or little activity present in the supernatant used as extract in the enzymatic assays was really not surprising in view of the formation of inclusion bodies in the IPTG induced transformant (Connaris et al; 1998). Previous studies have revealed that halophilic enzymes are usually unstable in chaotropic salts such as urea or guanidine hydrochloride (Hutcheon et al., 2005). The purified protein showed a specific activity of 163 ($\mu$moles PNPG/min/mg) versus 0.350 (μmoles PNPG/min/mg ) of *E.coli* Rosetta extract cells. This result shows a difference in activity of more than 90%, demonstrating that the transformant cells (TC23) can express the maltase from *H. walsbyi*. In addition, we demonstrated the difference in the expression level of the enzyme when it is in the presence or absence of the inductor isopropyl-D-thiogalactopyranoside (IPTG). The results show a difference of more than the 80% of the activity when the molecule IPTG is added to the culture growth of the transformant cells.

This work demonstrated that the recombinant protein have a significant enzymatic activity when it is resuspended in buffers solutions at 3M NaCl. Based on these preliminary experiments, we inferred that *H. walsbyi's* alpha glucosidase may be a possible membrane bound enzyme and not a cytoplasmic enzyme. Also with the use of

many different databases sources ("DAS" Transmebrane Prediction server, TMpred, HMMTOP) it was determined that the enzyme might have transmembrane helices (Fig. 18.). Also the TMpred and HMMTOP databases suggested a strongly preferred topology model that the N- terminus of the *H. walsbyi* putative alpha glucosidase enzyme is located outside the cell. Previous works have identified that the N domain residues of hipertermophilic archaea *S. solfatanicus* are essential for α-glucosidase substrate binding (Ernst et al., 2006). These results correlate with the previous experiments performed in *H. borinquense* and explained in Chapter 1 and can contribute to the understanding of catabolite repression in halophilic archaea. Having successfully expressing a halophilic recombinant enzyme, genetic experiments can be performed in the future to determine the nature of the regulation observed in *H. borinquense*.

The optimum temperature of the enzyme was 40 $^{0}$C and displayed optimal activity at a pH of 6.0 and 15% of NaCl. These results agree with the optimal growth conditions for *H. walsbyi* C23 strain which are: 45 $^{0}$C, pH 6.5 and 18% NaCl. Also, the pH at which the enzyme exhibited optimum activity is within the range of 5.0 to 6.0, which is typical for α-glucosidases (Constantino et al., 1990).

This study represents the first report of the expression, partial characterization and purification of an extremely halophilic alpha glucosidase from a halophilic archaeon. This work could have significant relevance in the food industry because halophilic microorganisms play an important role in the fermentation process; with the advantage

that they can produce some compounds that give the peculiar features of good flavor, taste and aroma to the fermented products (Margesin and Schinner 2001).

**General Conclusions**

1. Even though *H. borinquense* prefers glucose as carbon source, the three enzymes studied in this work (α-glucosidase, β-glucosidase, β-galactosidase) seem to be repressed at this growth condition.

2. Based on preliminary experiments, we inferred that *H. borinquense's* alpha glucosidase is a membrane bound enzyme because it presents a preference for NaCl as salt source.

3. Results indicate that the observed change in glycosyl hydrolase expression is related with the carbon source present in the media. These results might indicate that the halophilic archaea *H. borinquense,* uses the system of catabolite repression to regulate the genes involved in carbohydrate metabolism.

4. In this work we cloned and expressed an alpha glucosidase gene from the extremely halophilic archaea *Haloquadratum walsbyi.*

*5.* .*Haloquadratum walsbyi* alpha glucosidase enzyme presents its optimal levels of expression at 40 $^{0}$C, 15% (w/v) NaCl, and pH 6.0.

6. This study represents the first report of the expression, partial characterization and purification of a extremely halophilic alpha glucosidase from an archaeon.

**Recomendations**

1. To develop a strategy to purify the halophilic alpha glucosidase enzyme from *H. borinquense*. It might be necessary purify, express, and study the properties and physiological behavior of the recombinant protein and compare them to the native protein.

2. To corroborate the behavior of the studied enzymes (α-glucosidase, β-glucosidase and β-galactosidase) in response to salt preference, it is necessary to obtain and study in detail their protein sequences.

3. Westernblot analyses can be performed to test the possibility that the observed changes in enzyme levels resulted from changes in enzyme abundance rather than enzyme activity.

4. Additional experiments are also required to determine if the carbon catabolite repression in *H. borinquense* occurs at level of transcription, translation, or post translation modification.

5. In orden to obtain more information about the *H. walsbyi* alpha glucosidase protein and corroborate some of the data collected in this work it is necessarry to obtain the partial aminoacid sequence of the recombinant protein.

6. It could be necessary to study the substrate preference of the *H.walsbyi* alpha glucosidase enzyme.

7. It could be necessary to purify, express, and study the properties and physiological behavior of the H. walsyi alpha-glucosidase native protein and compare them to the recombinant protein.

8. Determine the stability of the *H. waslbyi* recombinant enzyme at diferents concentrations of NaCl, temperature and pH.

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