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Halophiles and their enzymes: Negativity put to good use

Shiladitya DasSarma and **Priya DasSarma**

Institute of Marine and Environmental Technology, Department of Microbiology and Immunology, University of Maryland School of Medicine, 701 East Pratt Street, Columbus Center, Baltimore MD 21202 USA

Abstract

Halophilic microorganisms possess stable enzymes that function in very high salinity, an extreme condition that leads to denaturation, aggregation, and precipitation of most other proteins. Genomic and structural analyses have established that the enzymes of halophilic Archaea and many halophilic Bacteria are negatively charged due to an excess of acidic over basic residues, and altered hydrophobicity, which enhance solubility and promote function in low water activity conditions. Here, we provide an update on recent bioinformatic analysis of predicted halophilic proteomes as well as experimental molecular studies on individual halophilic enzymes. On-going efforts on discovery and utilization of halophiles and their enzymes for biotechnology, including biofuel applications are also considered.

Introduction

Halophiles thrive from sea salinity (~0.6 M) up to saturation salinity (>5 M NaCl), and include Archaea, Bacteria, and Eukarya [1]. Many halophilic microorganisms have been isolated from diverse environments, ranging from artificial solar salterns, to natural brines in coastal and submarine pools, and deep salt mines. Some of the most commonly observed halophiles are those flourishing in salterns used for salt production, e.g. *Halobacterium* spp. (a misnomer, being members of the domain Archaea), *Salinibacter ruber* (a member of the Bacteroidetes phylum), and *Dunaliella salina* (green alga of the Chlorophyceae class) (Table 1). Halophilic microorganisms also have long been recognized as agents of spoilage of fish and meat preserved with solar salt and some varieties have been used for fermentation of protein-rich foods.

Over the past few decades, adaptation of halophilic microorganisms to their environment has been the subject of increasing interest, with methodology for culturing, manipulation, and genetic engineering steadily advancing. Our understanding of the adaptation of halophiles to high salinity includes several different mechanisms for balancing the osmotic stress of the external medium. Halophilic Archaea (Haloarchaea) primarily use a “salt-in” strategy,

Corresponding author: Prof. Shiladitya DasSarma, sdassarma@som.umaryland.edu, 1-410-234-8847 (voice), 1-410-234-8896 (fax). pdassarma@som.umaryland.edu(P. DasSarma)

accumulating concentrations of KCl equal to NaCl in their environment, and where examined, their enzymes tolerate or require 4-5 M salt [2]. In contrast, most halophilic Bacteria and Eukarya, largely use a “salt-out” strategy, excluding salts and accumulating or synthesizing *de novo* compatible solutes (e.g. glycine betaine and other zwitterionic compounds for Bacteria, and glycerol and other polyols for Eukarya) [3]. Among some halophiles, a combination of adaptive mechanisms may operate.

Early microbiologists addressing the adaptation of halophilic enzymes to high salinity discovered two primary features: a substantial number of protein charges and increased hydrophobicity [4]. Dissolved ions shielded electrostatic repulsions at low (<1 M) concentrations of salts and increased hydrophobic effects occurred at higher concentrations, from 4 M to saturating conditions. Roles for specific ion pairs were also sometimes suggested, e.g. in stabilizing active site regions or promoting subunit interactions. The combined effects of these forces were hypothesized to result in improved function in hypersaline conditions, where most non-halophilic proteins are inactivated by low water activity and limiting solvation, resulting in their denaturation, aggregation, and precipitation.

In the 1990’s, the availability of the first solved structure of a halophilic enzyme and a halophile genome sequence provided a much more detailed molecular perspective on halophilic adaptations than previously available [5-7]. Subsequently, over the next two decades, there has been a veritable explosion in studies of halophiles and their enzymes [8]. In this article, we review the key features of halophilic proteins and enzymes revealed from bioinformatic, structural, genetic, and biochemical studies over the past few years and address some potential applications to biotechnology.

Insights from bioinformatic analysis

The striking negativity of the halophilic proteome was first revealed by genome sequencing of *Halobacterium* sp. NRC-1 (Table 1) [6-9]. A unimodal distribution of protein isoelectric points (pI) with a mean of 5.0 and mode of 4.2 was observed, in stark contrast to all non-halophilic proteomes which possess bimodal distribution with acidic and basic proteins and an average pI very close to neutrality (Fig. 1). *Halobacterium* exhibited an excess of acidic (glutamic and aspartic acid) and a deficit of basic (lysine and arginine) amino acids. Excess negative charges were localized to the surface of modeled proteins, consistent with available structural work [5,9,10]. With subsequent sequencing of many additional genomes, bioinformatic studies confirmed the great dominance of acidic residues and a deficit of basic residues, especially lysine, for halophilic prokaryotes, but not necessarily halophilic eukaryotes (Table 1) [11].

For Haloarchaea, orthologous proteins from more than a dozen genomes have recently been identified via reciprocal BLAST analysis to generate haloarchaeal protein families (called Haloarchaeal Orthologous Groups or HOGs) [12*]. This investigation resulted in the finding of nearly 800 acidic protein clusters (conserved HOGs or cHOGs) present in all Haloarchaea examined, including a subclass of unique proteins found only within this group. This work clearly established the acidic nature of all conserved haloarchaeal proteins in contrast to their non-halophilic orthologs. Databases of haloarchaeal genomes and/or protein families

have been made available on dedicated websites, even while the number of halophile genomes has continued to grow [13-15].

In addition to their negative nature, halophilic and especially haloarchaeal proteins have been found to contain a slightly lower composition of bulky hydrophobic side chains (phenylalanine, isoleucine, and leucine) on their surface compared to small and borderline hydrophobic (glycine, alanine, serine, and threonine) amino acid residues [16]. These findings are consistent with increased flexibility and surface hydration of halophilic proteins, which may account for the observed similarities of halophilic proteins to psychrophilic proteins [8,17**]. In a detailed study comparing 15 homologous pairs of halophilic and non-halophilic proteins, general trends of increased acidic amino acids and reduced large nonpolar amino acids were recently confirmed [18]. Dipeptide analysis further resulted in the finding of halophilic signatures, useful in predicting halophilicity of proteins and designing enzymes with halophilic or halotolerant properties [19,20].

Bioinformatic studies of genomes of halophilic Bacteria resulted in the finding of predicted proteomes with moderate negativity, consistent with the existence of a combination of salt-in and salt-out adaptive strategies (Table 1). *Acetohalobium arabaticum* and *Salinibacter ruber*, isolated from marine salterns, harbor the most acidic proteomes reported for Bacteria, but are nevertheless considerably less acidic than for Haloarchaea. Two moderately halophilic Proteobacteria, *Halomonas elongata* and *Chromohalobacter salexigens*, contain predicted acidic proteomes, albeit less acidic than more extreme halophiles. In *Halanaerobium hydrogeniformans*, isolated from anaerobic sediments of hypersaline lakes, a slightly acidic proteome was recently predicted from the genome, raising questions about the reportedly high internal salt concentrations [21]. An interesting comparative study of two halophilic Proteobacteria, *Halorhodospira halophila* and *Halorhodospira halochloris*, revealed divergent results, with the proteome of the former being moderately acidic and the latter being less so (Table 1). The internal salt concentration was high in *H. halophila*, but low in *H. halochloris*, suggesting that different adaptive mechanisms operate [22**]. These recent results provide validation of the correlation between halophilic character and acidic nature of proteins, and suggest further that the degree of negativity of proteomes reflects the concentration of salts internally. Future studies are sure to further illuminate the diverse mechanisms of high salt adaptation operating among Bacteria.

Structural and biochemical characteristics

Structural and biochemical characterization of several halophilic enzymes has shown that enhancing solvation is the key requirement essential for maintaining solubility and activity of halophilic enzymes in low water activity, which can approach values as low as 0.75 in a saturated NaCl solution (Table 2) [8]. Under these extremely water-limited conditions, hydrogen bonds between negatively charged side chains and water molecules become critical to maintaining a stable hydration shell [5,23]. Other important factors for protein function in high salinity include increases in ion-pair networks, reduction of hydrophobic surface patches, and an unusually high number of ordered side chains. Low water activity conditions mimic aqueous-organic solvent mixtures, and consequently halophilic enzymes

generally retain considerable activity in organic media, making them potentially useful as industrial biocatalysts [8,24].

In an incisive series of studies, halophilic malate dehydrogenase enzymes and a non-halophilic homolog were compared to address the adaptive process, employing the power of structural biology [5,25]. Interestingly, when the *Salinibacter ruber* enzyme was included in the comparison, characteristics intermediate between the extremely halophilic Archaea and non-halophilic Bacteria became apparent [26]. The *S. ruber* enzyme was active at high salinity but also remained folded and active at low salt concentrations, and displayed acidic residues at the surface and both solvent-accessible and buried hydrophobic residues. These studies have suggested the existence of subtle tradeoffs between solubility, stability, and catalytic activity in the amino acid sequence of halophilic malate dehydrogenases.

In a study of a β-galactosidase from *Halorubrum lacusprofundi*, a cold-adapted halophilic Archaea from Antarctica, the enzyme was found to be active over an exceptionally broad temperature range (−5 to 70 °C) under optimal conditions (4-5 M NaCl or KCl) [27]. The enzyme was also found to display high stability and activity in aqueous solutions of alcohols, including methanol and ethanol. The basis for these remarkable biochemical characteristics was expanded by a combination of bioinformatic analysis and homology modeling, which predicted that certain amino acid residues at the enzyme surface and interior are critical for its halophilic character (Fig. 2) [17**]. Additional mutagenic studies of this model enzyme are likely to lead to valuable insights into adaptation to both temperature and salt extremes in the future.

Like all cells, halophiles accumulate K^+ ions, which are much less abundant in their environment, than Na^+ ions. Correspondingly, many (though not all) halophilic enzymes, reflect a preference for K^+ over Na^+ ions for optimal activity. However, while the size difference between Na^+ and K^+ ions is well-known, relatively few studies have addressed the molecular mechanisms responsible for the differential effects of these cations on enzyme activity. In a recent study on DNA ligase from *Haloferax volcanii*, K^+ ions were found to trigger catalytic activity by preferentially stabilizing the active site, and based on the predictions, the enzyme could then be mutagenized to improve performance in the presence of Na^+ ions [28].

The structural basis for salt resistance was also experimentally determined for a domain of DNA ligase from *Haloferax volcanii* [29]. Mutagenesis of surface residues resulted in interconversions between the obligately halophilic and less halophilic forms. Interestingly, despite the substantial bioinformatic evidence implicating surface amino acid biases for the halophilicity of proteins, analysis of the mutated DNA ligase showed that the effects of salt on the stability of this protein is largely independent of the total protein charge, but instead, appears to be correlated to the reduction of hydrophobicity of the accessible surface area.

Disulfide bonds have long been known to stabilize proteins, and in a recent study of a halophilic enzyme, their effects were addressed experimentally. An aspartic acid to cysteine mutation was introduced in a *Halobacterium* nucleoside diphosphate kinase, which requires high salt concentration for proper folding, resulting in the formation of a disulfide bond

between subunits [30*]. While the wild-type protein required at least 2 M NaCl for renaturation of the unfolded polypeptide, the mutated protein recovered activity at much lower salt concentrations, even at 0.1 M NaCl, while retaining a partially oligomeric state. These findings may provide a valuable approach for design of new halophilic enzymes active in hypersaline conditions.

In addition to structure and activity, salts may have profound effects on the folding of halophilic proteins and enzymes. In one study, a domain of ProtL of *Streptococcus magnus*, a model non-halophilic protein which binds to the κ light chain immunoglobulin, was converted into a "halophilic" variant by mutagenesis of 6 lysine residues to glutamic acid (Kx6E) [31*]. Surprisingly, however, the hydration dynamics of Kx6E did not differ substantially from ProtL, or from other previously studied variant proteins. This finding was interpreted to be a challenge to the dogma that halophilic proteins function through exceptional hydration. While reflecting unexpected results in a non-native system, this study underscores the need for additional research, especially through detailed mutagenesis and structure-function analysis. Such future studies will help to establish and refine the subtle rules governing halophilic characteristics.

Halophilic transformations for biotechnology

Properties of halophilic microorganisms and their negatively charged enzymes make them potentially very useful for biotechnology. Hypersaline brines in which halophiles flourish provide ideal conditions for carrying out many biotechnological transformations, due to their great abundance and exclusion of non-halophilic contaminants. Halophiles may serve as a source of many unique biomolecules, such as stable enzymes, biopolymers, and compatible solutes, and they may also be valuable for bioremediation and biofermentation processes, and other novel applications in agriculture and medicine [32].

Several recent studies have appeared on the conversion of plant and animal polymers in high salt conditions, including the production of biofuels. One strain of *Haloarcula* isolated from a Turkish salt mine showed considerable cellulolytic activity at high salinity, and when the cells were immobilized in sodium alginate polymer, hydrolytic activity was further enhanced [33]. Another strain, from Chinese salt lake soil, showed cellulolytic activity in the presence of non-polar solvents, and hydrolysis of alkali-pretreated rice straw by the halophilic enzyme could be used for bioethanol fermentation [34]. A *Halolactibacillus* species was found that transformed raw corn starch to products, which could be converted to bioethanol [35**]. The extremely halophilic green alga, *Dunaliella salina*, was shown to produce high lipid content suitable for biodiesel production [36**,37]. Other halophilic lipases which may potentially produce biodiesel have also been reported [38].

Chitin is another abundant biopolymer for which halophilic hydrolases have been demonstrated recently, following the report of chitinase genes in genome sequences [7]. A *Natrinema* strain harbors a serine protease capable of deproteinizing chitin-containing biomass at high salinity, including shrimp shell powder [39*]. *Haloferax mediterranei* interestingly displays both chitinolytic and polyhydroxyalkanoate biosynthetic capabilities [40,41**]. Genetic analysis demonstrated the involvement of chitin utilization and the novel

PHBV [poly(3-hydroxybutyrate-co-3-hydroxyvalerate)] polymer synthesis pathways, suggesting the potential for conversion of chitin into valuable bioplastics by this halophile.

Denitrification is another potential application for the metabolically versatile *Haloferax mediterranei*. This species can utilize nitrate and nitrite as nitrogen sources for growth in the presence of oxygen and by a denitrification pathway when oxygen is limiting [42]. Removal of nitrite and nitrate from contaminated brine could be shown using this species, utilizing nitrate respiratory enzymes [43*]. Additional efforts at deeper understanding of these conversions are being directed using transcriptomic studies [44].

Researchers have successfully identified individual halophilic microorganisms and consortia involved in hydrocarbonoclastic activity in oily saline soils [45] and via biofilm formation in crude oil and hypersaline brine mixtures [46]. Strains from the Persian Gulf coast capable of both hydrocarbon utilization and volatilization of toxic mercury were recently reported [47]. A *Haloferax* strain was found to produce surfactants that enhanced hydrocarbon degradation [48]. Additionally, an *Arhodomonas* strain was reported to have the capability to degrade aromatic compounds, including benzene and toluene [49].

Conclusion

It is anticipated that halophiles and their negatively charged enzymes will be put to good use and be of increasing value in future. Halophiles produce extraordinarily stable enzymes which function under conditions where conventional enzymes cease to function, denature, and precipitate. A host of recent studies have illuminated how halophilic enzymes manage to bind water tightly and maintain solvation and solubility in extremely high salinity and low water activity conditions. While future laboratory studies are sure to refine our fundamental understanding of halophilic enzymes at a mechanistic level, isolation of new halophiles from the environment will likely further expand the availability of valuable enzymes for industrial processes, e.g. catalysis in aqueous-organic solutions and fermentation of plant polymers in concentrated brines. Methods are also becoming available for directing the overexpression of halophilic enzymes and bioengineering novel pathways in extreme halophiles. Hypersaline conditions may provide distinct advantages for some processes, such as in agricultural, bioremediation, or biofuel applications, and extremely saline conditions may allow biotransformations to be conducted in the absence of strictly sterile conditions due to the growth inhibitory effects of brine on non-halophiles. Moreover, the increasing cost of freshwater will likely make brine the preferred medium of choice for many applications. As a result of these unique properties and capabilities, the importance of halophiles and their enzymes to both basic and applied research is likely to expand over the next few years.

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Highlights

- Halophiles produce stable enzymes that are active under high salt conditions
- Halophilic enzymes maintain solvation and solubility in low water activity
- Halophiles are valuable for industrial processes in organic solvents and brine
- Halophilic processes may be scaled up in absence of strictly sterile conditions

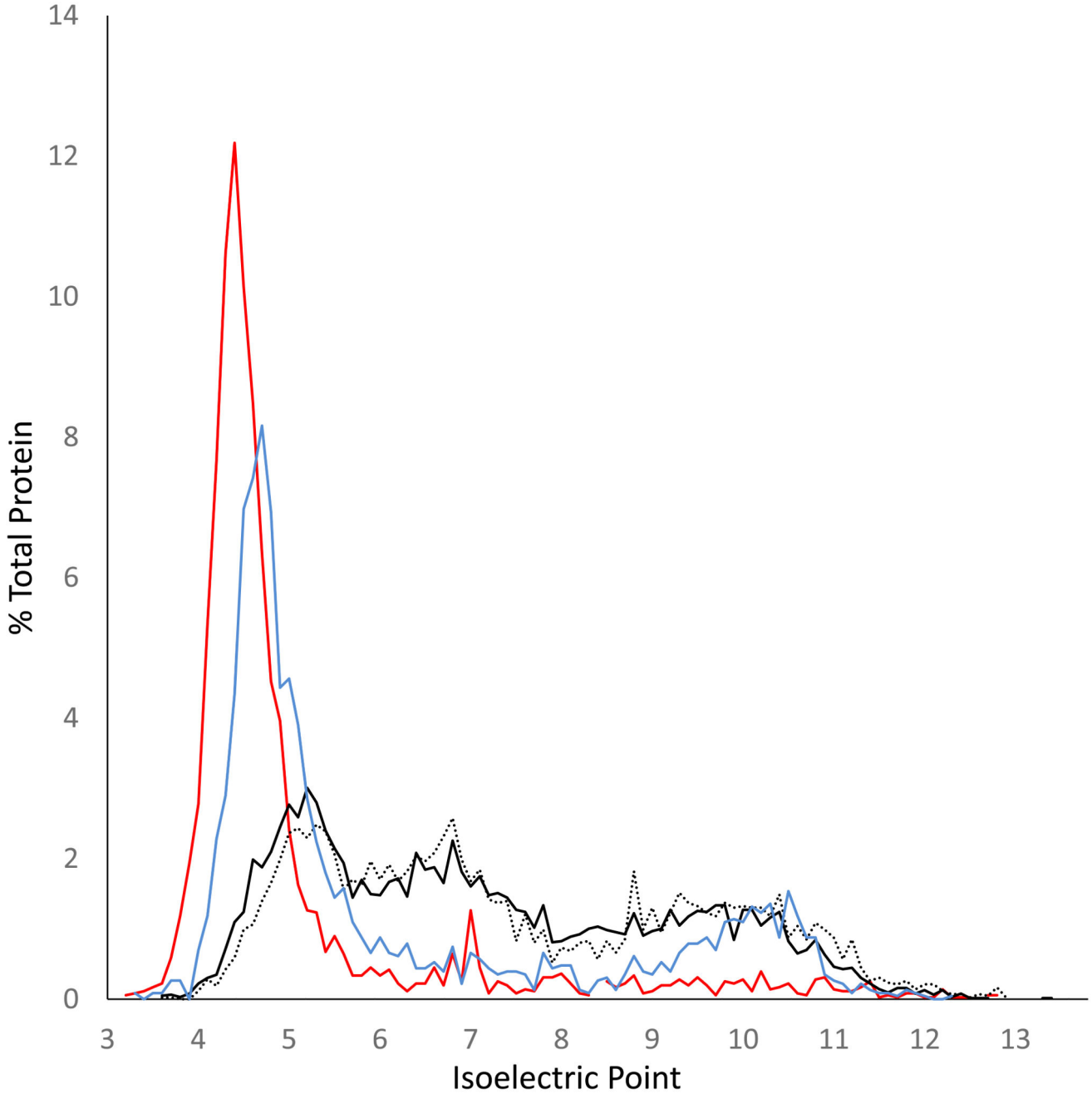

Figure 1. Distribution of protein isoelectric points predicted from genome sequences

Percent total protein is plotted versus pI in 0.1 increments for *Halorubrum lacusprofundi* (red), *Acetohalobium arabaticum* (blue), *Debaryomyces hansenii* (black line), and *Escherichia coli* (black dots).

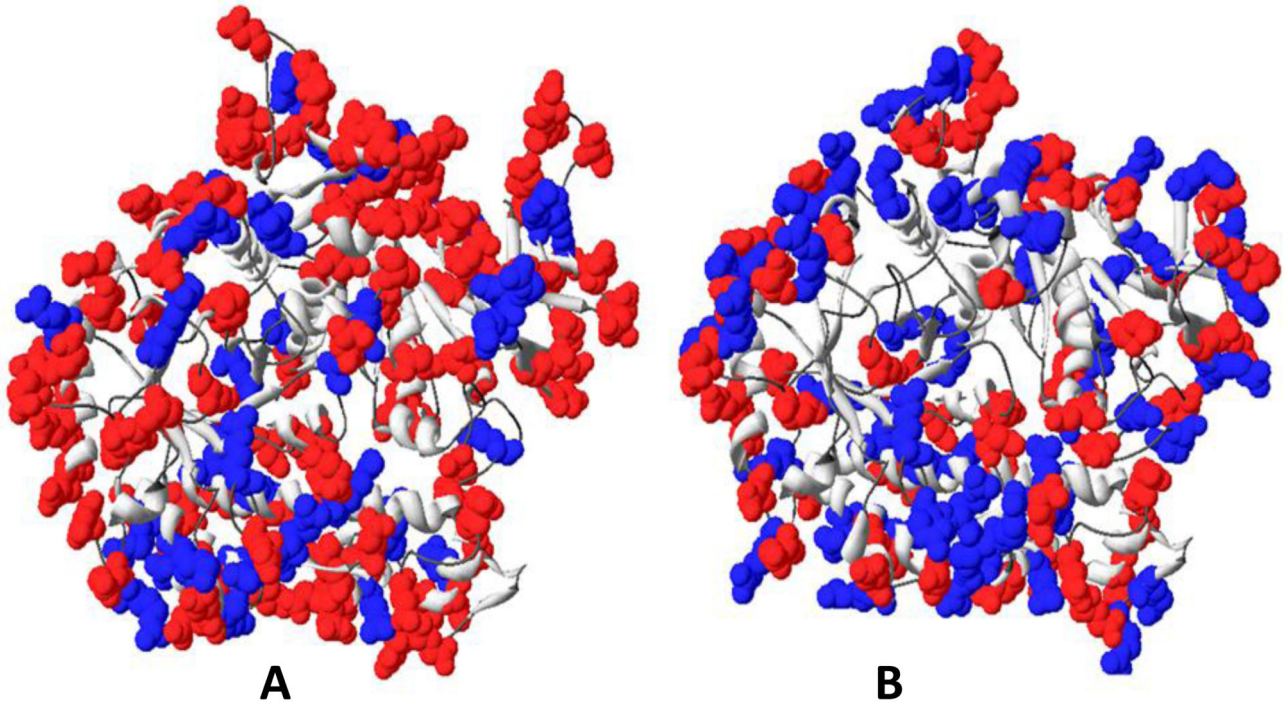

Figure 2. Comparison of surface acidic and basic amino acids in β-galactosidases

A model structure is shown for the enzyme from *Halorubrum lacusprofundi* (A) and crystal structure for that of *Thermus thermophilus* (B), with backbone in white in ribbon form and surface acidic (red) and basic (blue) groups shown using space-filling models. The net surface charges are −65 (A) and −4 (B).

Table 1
Representative halophilic microorganisms

Domain and Organism	Physiology and isolation	Mean pI*
Archaea		
Halobacterium sp. NRC-1	Laboratory model, phototrophic facultative anaerobe	5.0
Haloferax volcanii	Laboratory model, prototroph, from Dead Sea mud	5.0
Haloferax mediterranei	Versatile metabolism, PHA producer from Spanish saltern	4.8
Halorubrum lacusprofundi	Cold-adapted halophile from Antarctic lake	4.8
Bacteria		
Acetohalobium arabaticum	Methylotrophic homoacetogenic Fermicute from Arabat lagoon	5.9
Chromohalobacter salexigens	Anaerobic chemoorganotrophic Proteobacterium from Bonair	6.6
Halomonas elongata	Ectoine producing Proteobacterium from Bonaire solar saltern	6.3
Halanaerobium hydrogeniformans	Hydrogen producing haloalkalophilic Firmicute from Soap Lake	6.6
Halorhodospira halochloris	Anaerobic purple sulfur Proteobacterium, Wadi Nantrun lake	6.7
Halorhodospira halophila	Anaerobic purple sulfur Proteobacterium, Summer Lake mud	6.3
Salinibacter ruber	Heterotrophic Bacteriodetes from Spanish solar saltern	5.9
Eukarya		
Debaryomyces hansenii	Hemiascomycetous yeast from New Zealand Soil	6.9
Dunaliella salina	Unicellular microalga/Chlorophyceae common in salterns	NA
Wallemia ichthyophaga	Xerophilic Basidomycete from Slovenian saltern	7.2

* Calculated from NCBI protein database using EMBOSS 6.3.1 IEP program at Pasteur Institute. NA: not available.

Table 2

Halophilic proteins with solved structures

Enzyme	Source(s)
malate dehydrogenase	*Haloarcula/Salinibacter*
nucleoside diphosphate kinase	*Halomonas/Halobacterium*
α-amylase	*Halothermothrix*
carbonic anhydrase	*Dunaliella*
glucose dehydrogenase	*Haloferax*
dihydrofolate reductase	*Haloferax*
alkaline phosphatase	*Halobacterium*
β-glucosidase	*Halothermotrix*
catalase/peroxidase	*Haloferax*
RNase H1	*Halobacterium*
D-mannonate dehydratase	*Chromohalobacter*