Halophilic bacteria and their compatible solutes – osmoregulation and potential applications

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Halophilic bacteria grow over an extended range of salt concentrations. Their metabolic patterns are distinct compared to their terrestrial counterparts and obligate halophiles. The possibility of rapid adjustment to changes in the external salt concentration makes them potential candidates for bioprocessing. Halophilic bacteria produce enzymes, metabolites, solutes, exopolysaccharides and pigments which have high commercial value. However, compared to the obligate halophilic archaea, they have been less studied with only a few reports available on designing the production processes.

Compatible solutes are the highly soluble, low-molecular weight organic compounds that make halophilic bacteria versatile in their adaptation to salinity. Halophilic bacteria either take in the solutes from the medium or synthesize them *de novo*. Compatible solutes include amino acids, carbohydrates or their derivatives, sugars and polyols. In addition to their stabilizing effects, they offer a multitude of physiological and potential biotechnological applications ranging from stabilizers of biomolecules, stress-protective agents to therapeutic agents and cosmetic actives. Exploring the significant applications of compatible solutes could be an attractive proposition towards commercialization. Moreover, it would enlighten the bases of adaptations of moderately halophilic bacteria.

Keywords: Betaine, compatible solutes, ectoine, halophilic bacteria, osmoregulation.

HALOPHILES are microorganisms that adapt to moderate and high salt concentrations. They are found in all three domains of life: *Archaea*, *Bacteria* and *Eukarya*. Halophilic bacteria grow over an extended range of salt concentrations (3–15% NaCl, w/v and above), unlike the truly halophilic archaea whose growth is restricted to high saline environments[1].

Water availability in microorganisms is inversely proportional to the concentration of dissolved salts[2]. Halophilic archaea usually employ a continuous influx of ions like K^+ in order to balance the high salt environment outside the cell. In such cases the intracellular protein machinery is dependent on high salt concentrations for function and stability. On the other hand, halophilic bacteria usually synthesize or accumulate compatible solutes to maintain the osmotic equilibrium in response to the high-salt external environment. These organic molecules are compatible with the intracellular machinery even at molar concentrations and hence the name[3]. They maintain the cell volume, turgor and electrolyte concentrations within the cell system. As a result, an appropriate hydration level of the cytoplasm is achieved and cell growth can proceed under osmotically unfavourable conditions[4].

Compatible solutes are low-molecular weight osmoregulatory compounds which are highly water-soluble sugars, alcohols, amino acids, betaines, ectoines or their derivatives. In addition to their stabilizing effects, they are used as salt antagonists, stress protective agents, moisturizers and therapeutics. They stabilize enzymes, DNA and whole cells against stresses such as freezing, drying and heating. They increase freshness of foods by stabilizing components. Induction of osmolytes in cells can increase protein folding and thereby improve salt tolerance which could be useful in agriculture and xeriscaping[5,6].

Diversity and perspectives of halophilic bacteria

Halophiles

Halophiles are a group of microorganisms that live in saline environments and in many cases require salinity to survive. Halophiles include a great diversity of organisms, like moderately halophilic aerobic bacteria, cyanobacteria, sulphur-oxidizing bacteria, heterotrophic bacteria, anaerobic bacteria, archaea, protozoa, fungi, algae and multicellular eukaryotes. Microorganisms that are able to grow in the absence as well as in the presence of salt are designated as halotolerant and those that are able to grow above approximately 15% (w/v) NaCl (2.5 M) are considered extremely halotolerant[7,8].

According to Kushner[9], many marine organisms are slight halophiles (with 3% w/v NaCl in sea water). Moderate halophiles optimally grow at 3–15% w/v NaCl; extreme halophiles at 25% w/v NaCl (halobacteria and

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halococci) and borderline extreme halophiles require at least 12% w/v salt.

Moderately halophilic bacteria

This group of bacteria has been reviewed extensively by Ventosa *et al.*[10]. The occurrence of nonpigmented halotolerant bacteria is said to be first mentioned in 1919 by LeFevre and Round[11] in their study on microbiology of cucumber fermentation brines. Halophilic bacteria are found in a variety of salt environments like marine ecosystems, salted meat, salt evaporation pools and salt mines. Most of the important groups of bacteria are able to live in concentrations up to about 15% salt and others can adapt to conditions even at higher salt concentrations[12]. They form a versatile group and are adapted to life at the lower range of salinities and have the ability to adjust rapidly to changes in the external salt concentration.

Halotolerance

Halotolerance is the adaptation of living organisms to conditions of high salinity. Proteins of halophilic microorganisms contain an excess ratio of acidic to basic amino acids and are resistant to high salt concentration. Proteins in extreme halophiles have structure–function stability only in the presence of salt and their enzymes require salts for activity. Surface negative charges prevent denaturation and precipitation of proteins at high salt concentrations[8]. Adaptation to conditions of high salinity has an evolutionary significance. The concentration of brines during prebiotic evolution suggests haloadaptation at earliest evolutionary times[13]. An understanding of halotolerance can be applicable to areas such as arid-zone agriculture, xeriscaping, aquaculture or remediation of salt-affected soils. In addition, knowledge of halotolerance involving osmotic changes can also be relevant to understanding tolerance to extremes in moisture or temperature[14].

Osmoregulation

Many microorganisms respond to increase in osmolarity by accumulating osmotica in their cytosol, which protects them from cytoplasmic dehydration[15]. Osmophily refers to the osmotic aspects of life at high salt concentrations, especially turgor pressure, cellular dehydration and desiccation. Halophily refers to the ionic requirements for life at high salt concentrations. According to Rothschild and Mancinelli[16], organisms live within a range of salinities essentially from distilled water to saturated salt solutions.

Halophilic microorganisms usually adopt either of the two strategies of survival in saline environments: ‘compatible solute’ strategy and ‘salt-in’ strategy[10]. Compatible solute strategy is employed by the majority of moderately halophilic and halotolerant bacteria, some yeasts, algae and fungi. In this strategy cells maintain low concentrations of salt in their cytoplasm by balancing osmotic potential through the synthesis or uptake of organic compatible solutes. Hence these microorganisms are able to adapt to a wide range of salt concentrations. The compatible solutes include polyols such as glycerol, sugars and their derivatives, amino acids and their derivatives, and quaternary amines such as glycine betaine and ectoines.

The salt-in strategy is employed by true halophiles, including halophilic archaea and extremely halophilic bacteria. These microorganisms are adapted to high salt concentrations and cannot survive when the salinity of the medium is lowered. They generally do not synthesize organic solutes to maintain the osmotic equilibrium. This adaptation involves the selective influx of K^+ ions into the cytoplasm. All enzymes and structural cell components must be adapted to high salt concentrations for proper cell function.

Potential of halophilic bacteria for biotechnology

The industrial and environmental applications of halophilic microorganisms have been reviewed by Oren[17]. The review highlights the salient features of halophiles, including their highly successful applications like β-carotene production by *Dunaliella* and ectoine synthesis using *Halomonas* and other moderately halophilic bacteria. The potential use of bacteriorhodopsin, the retinal protein proton pump of *Halobacterium* is being explored in photochemical processes. Other possible uses of halophilic microorganisms, as discussed in the review, include treatment of saline and hypersaline wastewaters and production of exopolysaccharides, poly-β-hydroxyalkanoate bioplastics and biofuel.

Halophilic bacteria offer potential applications in various fields of biotechnology[18]. Although moderately halophilic bacteria have many industrial applications, only a few studies have been carried out concerning this aspect. These microorganisms can be used as a source of metabolites, compatible solutes and other compounds of industrial value. Novel halophilic biomolecules may also be used for specialized applications, e.g. bacteriorhodopsin for biocomputing, pigments for food colouring and compatible solutes as stress protectants[8]. Biodegradation of organic pollutants by halophilic bacteria and archaea has been recently reviewed[19]. These microorganisms are good candidates for the bioremediation of hypersaline environments and treatment of saline effluents. Understanding the degradation process would also shed light on the enzymes involved and on the regulation of metabolism.

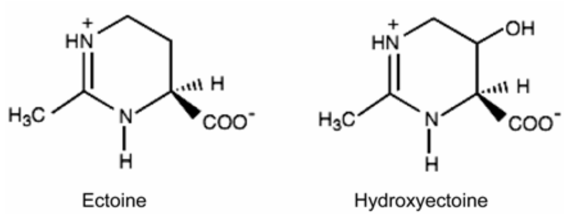
Halophilic bacteria are a potential source of extracellular hydrolases like proteases with a wide array of industrial applications. These enzymes exhibit stability over a range of saline conditions[20]. Halophilic bacteria constitute excellent models for the molecular study of osmoregulatory mechanisms[10]. Naturally halotolerant plants or microorganisms could be developed into useful agricultural crops or fermentation organisms. This has possible application in agriculture, where conventional agricultural species could be made more halotolerant by gene transfer from naturally halotolerant species. *Arabidopsis thaliana* transformed with a choline oxidase gene from *Arthrobacter globiformis* has been reported to have a significantly improved tolerance of salt stress[21]. This field offers a lot of scope for research and development.

Compatible solutes

Compatible solutes are low-molecular weight osmoregulatory compounds, including highly water-soluble sugars, alcohols, amino acids, betaines, ectoines or their derivatives (Table 1)[5,10,21]. Compatible solutes are useful as stabilizers of biomolecules and whole cells, salt antagonists or stress-protective agents. Due to their stabilizing effects, they can be used for various research and industrial applications. Moderate halophiles are expected to produce polar uncharged and zwitterionic solutes and halo-thermophilic microbes produce anionic compatible solutes.

Compatible solutes have protein-stabilizing properties that help in the proper folding of polypeptide chains[22]. Due to their stabilizing effect on protein molecules they are also sometimes referred to as chemical chaperones[23]. Conformational shift of protein towards folded, native-like states induced by preferential exclusion of the solute is responsible for the chaperone-like effects[24]. Compatible solutes exert their effect through changes in solvent structure and/or subtle changes in the dynamic properties of the protein rather than by changing the structure of the protein itself[25]. Compatible solutes also interact with nucleic acids and can influence protein–DNA interactions[26,27].

Ectoine



Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is one of the most common osmotic solutes in the domain bacteria. It was first discovered in the haloalkaliphilic photosynthetic sulphur bacterium, *Ectothiorhodospira halochloris*, but later a great variety of halophilic and halotolerant bacteria were found to produce this compound, often together with its 5-hydroxy derivative[16]. Ectoines are common in aerobic heterotrophic eubacteria. The entry molecule into ectoine biosynthesis is aspartate semialdehyde, which is an intermediate in amino acid metabolism. The aldehyde is converted to L-2,4-diaminobutyric acid, which is then acetylated to from *N*γ-acetyldiaminobutyric acid (NADA). The final step is the cyclization of this solute to form ectoine. Ectoine synthesis is carried out by the products of three genes: *ectABC*. The *ectA* gene codes for diaminobutyric acid acetyltransferase; *ectB* codes for the diaminobutyric acid aminotransferase and *ectC* codes for ectoine synthase[5].

Ectoines have gained much attention in biotechnology as protective agents for enzymes, DNA and whole cells against stresses such as freezing, drying and heating. In recent years additional properties of interest were found for ectoine. It is claimed that it counteracts the effects of UV-A-induced and accelerated skin ageing, and therefore, is being used as a dermatological cosmetic additive in moisturizers for the care of aged, dry or irritated skin. Ectoine also inhibits aggregation of Alzheimer's

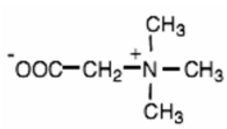
Table 1. Distribution of some major compatible solutes in prokaryotes

Compatible solutes	Occurrence
Ectoine	*Halomonas elongata*, *Ectothiorhodospira halochloris*, *Halomonas boliviensis*, *Brevibacterium epidermis*, *Chromohalobacter israelensis*, *Chromohalobacter salexigens*
Hydroxyectoine	*H. elongata*, *Nocardiopsis halophila*, aerobic heterotrophic bacteria
Betaine	*Actinoployspora* sp., *Halorhodopira halochloris*, *Thioalkalivibrio versutus*
Proline	*Streptomyces*, halophilic/halotolerant *Bacillus* strains
α-Glutamate	Marine bacteria, some methanogenic archaea
Mannosylglycerate	*Methanothermus fervidus*, *Pyrococcus furiosus*, *Rhodothermus marinus*, *Thermus thermophilus*, *Pyrococcus furiosus*, *Thermococcus* spp.
Diglycerol phosphate	*Archaeoglobus fulgidus*
Mannosylglyceramide	*Rhodothermus marinus*
Glucosylglycerate	*Agmenellum quadruplicatum*, *Erwinia chrysanthemi*, *Stenotrophomonas maltophilia*
Trehalose	*Pyrobaculum aerophilum*, *Thermoplasma acidophilum*, *Actimopolyspora halophila*, *Rubrobacter xylanophilus*
Sucrose	*Anabaena* (blue green algae), *Nitrosomonas europaea* and proteobacteria
Mannitol	*Pseudomonas putida*

β-amyloid, and recently, a clinical trial was initiated to test its efficacy in inhalations against bronchial asthma[17].

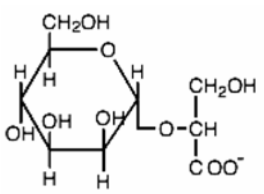
Ectoines are used for increasing the stability and freshness of foods by stabilizing food components. Ectoines also find applications in the treatment of the mucous membranes of the eye. Ophthalmologic preparations containing these molecules are useful for eye treatment to decrease the dryness syndrome. Introduction of ectoine and its derivatives into preparations for oral care has also been suggested[6].

Betaine



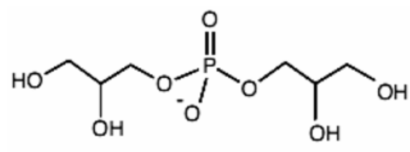
Betaines are the compatible solutes occurring in halophilic phototrophic bacteria, chemotrophic bacteria and archaebacteria. They have therapeutic potential for the treatment and prophylaxis of adipose infiltration of the liver, which is the initial stage of cirrhosis[6]. Betaines decrease side effects of anti-inflammatory preparations. Their anticoagulant properties prevent thrombus formation and decrease the probability of heart attacks, infarctions and strokes[28]. They are useful in PCR amplification of GC-rich DNA templates to increase product yield and specificity[5]. Betaine was shown to be a more effective cryo-protectant than serum albumin or trehalose/dextran, particularly under conditions stimulating long-term storage[29].

Mannosylglycerate



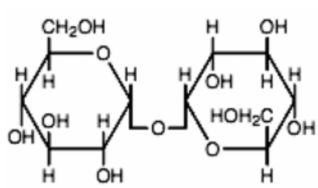
Mannosylglycerate (MG) is a novel compatible solute widely found in the halotolerant *Methanothermus fervidus*, *Pyrococcus furiosus* and *Rhodothermus marinus*. This compound has also been detected in many hyperthermophilic archaea, where it accumulates concomitantly with increasing salinity of the medium[30]. Although present in many red algae, the apparent restriction of MG to thermophilic bacteria and hyperthermophilic archaea led to the hypothesis that it plays a major role in thermal adaptation[21]. In *R. marinus*, there is a direct condensation of GDP-mannose and D-glycerate to form MG catalysed by mannosylglycerate synthase[5]. Possible applications are utilization as protectants for enzymes against physical or chemical stress, as additive in PCR, and excipient in pharmaceuticals.

Diglycerol phosphate



Diglycerol phosphate accumulates under salt stress in the hyperthermophile *Archaeoglobus fulgidus*. This new compatible solute is a potentially useful protein stabilizer, as it exerted a considerable stabilizing effect against heat inactivation of various dehydrogenases and a strong protective effect on rubredoxins (with a fourfold increase in the half-lives) from *Desulfovibrio gigas* and *Clostridium pasteurianum*[1].

Trehalose



The non-reducing glucose disaccharide, trehalose is used by organisms to counteract drying, but it also serves as an osmolyte[5]. It occurs in a wide variety of organisms, from bacteria and archaea to fungi, plants and invertebrates. Trehalose is not only useful as a cryoprotectant for the freeze–drying of biomolecules, but also for long-term conservation of microorganisms, as the membrane structure is preserved in the presence of this disaccharide[21].

Production of compatible solutes

For practical applications, reasonable quantities of compatible solutes have to be generated either *in vitro* or *in vivo*. A novel bioprocess for the production of ectoine from *Halomonas elongata* called 'bacterial milking'[31] has been the basis of the German biotechnology company Bitop, which develops products from osmolytes. After a high-cell-density fermentation, cells are fivefold concentrated using cross-flow filtration. Bacteria in high concentrations of NaCl are subjected to osmotic shock by transferring the cell biomass to low osmolarity medium, where they rapidly excrete the now excess solutes in the medium to maintain the osmotic equilibrium. After each dilution step, the medium (containing the solutes) has to be removed prior to re-exposure to high salt. Subsequent reincubation in a medium of higher salt concentration results in the resynthesis of these compatible solutes. The process could be repeated several times after a defined generation time. After nine repetitions, 155 mg ectoine/g cell dry wt/cycle was produced.

A process comprising two fed-batch cultures for the production of compatible solute ectoine and biopolyester poly(3-hydroxybutyrate) by a moderate halophile, *Halomonas boliviensis* has been reported[32]. The co-production process as described has been proposed for lowered production costs of the respective molecules. Application of statistical design involving response surface methodology allowed a quick optimization of medium components for ectoine production by *H. boliviensis*[33]. An overall ectoine volumetric productivity of 6.3 g/l/day was obtained. The optimized medium also showed improvement in ectoine productivity when used in fed-batch fermentation. An alternative, economically viable production method was proposed by demonstrating that a metabolic bottleneck for ectoine production in the non-halophilic recombinant *Escherichia coli* DH5α can be relieved by coexpression of deregulated aspartate kinase from *Corynebacterium glutamicum*[34].

Product recovery and purification

For the determination of endogenous compatible solute accumulation in a study by Teixidó *et al.*[35], cell suspensions were centrifuged; cells were harvested and freeze-dried. Subsequently, cell material was extracted using the method described by Kunte *et al.*[36] for quantitative analysis with extraction mixture (methanol/chloroform/water 10 : 4 : 4 by volume) by vigorous shaking followed by the addition of equal volumes of chloroform and water. Phase separation was enhanced by centrifugation. The hydrophilic top layer containing compatible solutes was recovered and analysed by HPLC.

For the recovery of ectoines from the product solution[31], the pH of the solution was lowered with HCl to permit ectoines in their cationic form. Ectoines were purified using cation exchange resin packed in a column and subsequently eluted with NaOH. The ectoine fraction was marked by increased UV absorbance (at 230 nm). The recovered ectoines can be subsequently crystallized from water.

Several analytical methods have been established for the detection of compatible solutes and further quantification using HPLC analysis. Purification of compatible solutes relies on chromatographic steps. Ectoine and hydroxyectoine concentrations can be determined by HPLC analysis[37]. The quality of purified ectoines can be controlled using NMR measurement[38] as well as isocratic HPLC and FMOC–HPLC techniques. A combination of anion-exchange chromatography and pulse amperometric detection is a sensitive method that can detect osmolytes such as ectoine after hydrolytic cleavage of the pyrimidine ring[39]. Various methods of characterization of organic compatible solutes of halotolerant and halophilic microorganisms by NMR methods have been given by Roberts[40].

Concluding remarks

Halophilic bacteria are able to survive in a wide range of salinities. Detailed study is needed to exploit the potential applications of these microorganisms for their commercial viability. Moreover, it would be interesting to study the properties and structures of these microorganisms and their molecules in order to investigate halophilic adaptations. Compatible solutes from these microorganisms can be used for a wide range of applications, including protein and/or enzyme stabilizers, cosmetic actives, therapeutic agents, bioremediation and also in improving the salinity tolerance of crop plants by gene transfer. Overall, the review has generated information on compatible solutes from halophilic bacteria and probed into their potential applications, thereby contributing towards the limited information available for the industrial applications of halophilic bacteria and their compatible solutes.

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