ECTOINES: A NEW TYPE OF COMPATIBLE SOLUTES WITH GREAT COMMERCIAL POTENTIAL

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Summary

Extremophilic microorganisms are, for many reasons, fascinating. They belong to the oldest forms of living on Earth, the *Archaea*, and might be seen as an example for understanding the various strategies of survival under high or low temperature as well as extreme pressure and drastic salt concentrations. More and more, researchers realize that the microorganisms are not only interesting from a scientific point of view but they also have great economical potential.

While there is already a great commercial use of the old compatible solutes betaine and trehalose, the market for the new compatible solutes such as ectoines is just beginning to emerge in areas such as food processing or fermentation of microorganisms. However, ectoine has already started its career as a new and hopefully revolutionizing substance for skin protection in the cosmetic market.

With all the possible applications, ectoines will not only be very successful commercially, it will hopefully also be a starting point for more research activities and commercial exploration into the interesting and promising world of adaptation to stress situations so nicely managed by extremophilic microorganisms.

1. Introduction

For literally thousands of years, salt solutions have been used for preserving meat, fish, and vegetables. It was long believed that no life could exist at salt concentration in access of 10% or 100 g sodium chloride per liter water. However, this view had to be changed at the beginning of of twentieth century when LeFevre and Round found microorganisms in salt solution of up to 150 g L^{-1}. It took almost 50 years to revive the research on such extreme microorganisms, which by that time were called by Macelroy extremophilic (from the Greek word *philos* for lover). Certainly, not all love the extreme environment. Very often, they are very difficult to grow and a lot of experience is needed to reach sufficient cell densities for the analysis of the special entities to be found in the cells. However, at least they sustain the drastic conditions and survive. Surprisingly, they live not only in water of high salt concentration, but some are also adapted to other extreme conditions such as high (thermophilic) or low temperatures.

It is certainly justified and clear cut to define a halophilic organism to require for an optimal growth salt concentrations well above the marine concentration of about 3%. On the contrary, the definition of halotolerant imposes some problems, since the degree of tolerance depends to some extent on the composition of the growth medium. By accumulation of protectants from the environment it is certainly possible for some bacteria to “acquire” halotolerance. Therefore a wide spectrum of tolerance is possible. By all means it is justified to say that the halophilic/halotolerant microbial world is not less diverse than the freshwater environment. In fact, they have evolved or remained in many different groups of organisms.

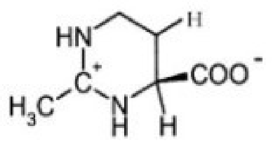
Microorganisms do not possess systems for an active transport of water. Therefore osmosis is very much governing the cellular water content as well as the turgescence. Most eubacteria as well as algae, fungi, or yeast that are capable of growing at high salt or solute concentrations achieve osmotic balance across the cell membranes by two different strategies. The first mechanism, which has been discovered and is typical for *Halobacteriaceae* uses potassium chloride to achieve osmotic equilibrium. This simple method, however, needs a substantial number of physiological changes and shows a relatively narrow adaptation for a specific environment. This mechanism is used by the group of halobacteria (Archaea).

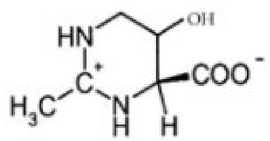
The second mechanism is widely used in eubacterial halophiles and employs the accumulation of nonionic organic molecules of low molecular weight to counterbalance the decrease water activity due to an increase in the salt concentration in the environment. These small molecules are called compatible solutes. They are derived from different molecule classes such as polyols, sugars, betaines, certain amino acids, and their derivatives. This mechanism of using compatible solutes has the great advantage of being much more flexible and suitable over a wide range of salinity. It is of great advantage that it is still possible to a “normal” salt-sensitive enzymatic machinery.

2. Ectoine—a Compatible Solute in Halophilic Microorganisms

Halophilic microorganisms include a wide ranges of microbes such as some archaea, cyanobacteria, and the green alga *Dunaliella salina*. We here, however, refer only to bacteria such as the Gram-negative and moderately halophilic *Halomonas elongata*. While most prokaryotes use glycine betaine as a very effective osmoticum, in an investigation of about 450 cultures from specific saline habitats it was found, that a previously described new class of compatible solutes, the ectoines, was predominantly found under such salt conditions.

Ectoines were originally found by Galinski in *Ectothiorhodospira halochloris*, an extremely halophilic phototrophic eubacterium. Later on the substance was also found in various moderately halophilic bacteria. Ectoines are widespread among halophilic and halotolerant eubacteria of which ectoine as 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid is the most prominent to be found. The hydroxy derivative, often found as a minor component in *Proteobacteria* seems to be of greater importance in Gram-positive eubacteria.





Ectoine®

molecular weight	142,2 g/mol
chemical formula	$C_6H_{10}N_2O_2$
microorganism	*Halomonas elongata*
storage	20 °C
melting point	280°C
solubility in water	4,0 mol/l
solubility in methanol	0,3 mol/l
pH range	1-9
opt. rot. Alpha-D-20°	+140
CAS-Nr.	96702-03-3

Hydroxyectoine

molecular weight	158,2 g/mol
chemical formula	$C_6H_{10}N_2O_3$
microorganism	*Marinococcus M52*
storage	20 °C
melting point	280°C
solubility in water	4,2 mol/l
solubility in methanol	0,05 mol/l
pH range	1-9
opt. rot. Alpha-D-20°	
CAS-Nr.	117229-60-4

Figure 1. Chemical structure and characteristics of ectoine and hydroxyectoine

The biosynthetic pathway of ectoine in the phototrophic bacterium *Ectothiorhodospira halochloris* has been proposed with the help of ^{13}C-labelling techniques and could be confirmed on enzymatic grounds. The synthesis of ectoine proceeds via aspartic semialdehyde and requires three additional enzymes: diaminobutyrate transaminase, diaminobutyrate acetylase, and Nγ-acetyldiaminobutyrate dehydrogenase. For the further formation of hydroxyectoine either a hydroxylase is needed or an alternative pathway with erythro-β-hydroxyaspartate might be used. The fact that relatively few and common enzymatic reactions form the basis of the biosynthesis of these two novel solutes probably explains why ectoines are so widespread among halophilic bacteria.

3. Industrial Production of Ectoines

Since ectoine could originally only be found in such small quantities, much of the research about these novel substances was hampered. Especially in the light of the production costs, at that time there was no hope for any immediate commercial use. Because there were some rather interesting results already received by testing chemically synthesized ectoine in possible cosmetic applications, the search began for a microorganism suitable for a large-scale biotechnological production of ectoines. It was thought, that the best organism to be found would have a broad salt tolerance as well as responding to a hypo-osmotic shock by rapid release of the accumulated solutes. These features had previously been reported for some halotolerant and halophilic bacteria. In addition the ectoine-producing bacteria should be also able to rapidly restore the amount of the compatible solute ectoine in the cells after being transferred to a hyperosmotic medium.

All these requirements were almost ideally fulfilled by the halophilic Gram-negative bacterium *Halomonas elongata* originally being isolated from a solar saltern. The natural property of the organism is to adapt to a wide range of salinities. A slow increase of salinity due the evaporation as well as fast dilution due to rainfall or flooding of evaporation ponds with sea water are typical events for an environment of fluctuating salinity. Therefore the microorganism was used in the development of a novel production process. Ectoine was the first molecule produced by such “bacterial milking."

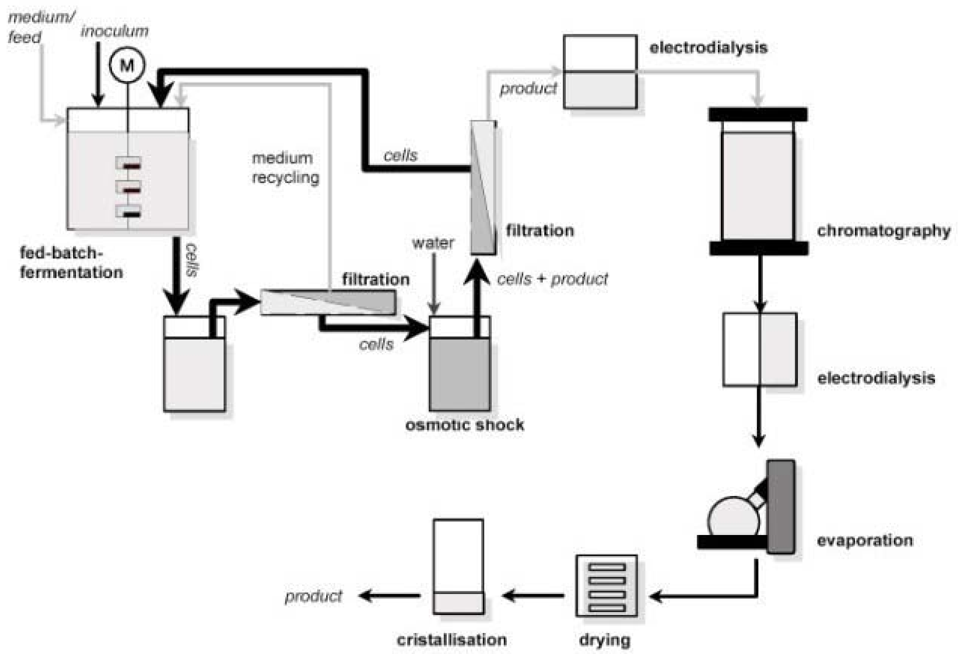


Figure 2. Process diagram for the production and purification of compatible solutes by using the platform technology “bacterial milking"

For the production of ectoine and hydroxyectoine, bitop AG uses the moderately halophilic and naturally occurring bacterium *Halomonas elongata*. Depending on the product wanted, the strain is grown at temperatures between 25 °C and 40 °C in a concentration of 15–20% salt (sodium chloride). The fermentation is performed in a 1 000-L as well as a 3 500-L scale fermentation vessel. At high-density fermentations we routinely reach an optical density of 60 and more. This corresponds to production concentrations between 8 and 12 g L^{-1} ectoine. In the process of coproduction of ectoine and hydroxyectoine, the total yields are somewhat less, but compared to other established reasonably good fermentation processes.

At our plant harvesting cells is usually done three times a week. Prior to a hypo-osmotic shock (for example from 15% salt to 3%) the volume of the culture is reduced by a crossflow filtration to about 20 per cent of the original volume. After the osmotic down shock ectoine is released and then separated from the cell mass. The bacteria are returned to the fermenter for the next round of fermentation while the product solution is further purified by electro-dialysis, chromatography, filtration, evaporation, freeze-drying and crystallization. At the end of the production line we are able to isolate tons of ectoine or hydroxyectoine per year of various degrees of purity. By a special and patented purification procedure, we are even able to isolate ectoine of very high purity of up to more than 99 per cent purity. Each lot receives an extensive protocol of analysis, which includes a HPLC analysis.

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Biographical Sketches

Georg Melmer is CEO of bitop AG, a German biotechnology company. He received his master's degree in chemistry as well as in psychology at the Ruhr-University of Bochum, Germany. There he also received his PhD in biochemistry. After working two years for a large pharmaceutical company in Germany, he returned to science and did postdoctoral work at the Hospital for Sick Children in Toronto, Canada. Here he was part of the team, which cloned the gene for cystic fibrosis. Later on he worked three years at the University College London at the Department of Psychiatry on the genetics of psychiatric disorders. After some more time at the University of Bochum, he joined a biotech company as scientific director and another one as managing director. In March 2000, he joined bitop AG as CEO. He is a coauthor of over 60 scientific papers and patents. Since also holding an MBA-like degree, he published over 100 articles about stock companies.

Thomas Schwarz is the CSO of bitop AG, a German biotechnology company. He received his master's degree as well as his PhD in biochemistry at the University of Witten/Herdecke, Germany. In 1993, together with the supervisor of his PhD, Prof. Bartholmes, he was a cofounder of bitop AG and served as head of research and development until he became the CEO in 1997. In March 2000, he became a CSO at bitop. He has a longstanding interest in extremophilic microorganisms and fermentation technology. He is a coauthor of some 20 scientific papers and patents.