1 Introduction

Within the group of extremophiles, halophilic microorganims developed specific cellular mechanisms to protect their interior processes and chemistry against the ruling environmental stress. Their natural saline habitats mainly change ionic strength by weather-reliant desiccation and irrigation. Such osmotic changes strongly affect the microorganisms by osmotic gradient between cell turgor and environment, and can lead to cell damage and death. To counteract this destructive influence, the halophilic microorganisms use short- and long-term adaptations, providing best possible flexibility at shock situations and thus increasing survivability [1].

In biotechnological application, this specific applicability is used to produce compatible solutes, amongst other valuable compounds in stress-related bioprocesses. Despite the stimulation of compatible solute production, the environmental stress affects cellular state and cell membranes, leading to heterogeneities and thus diminishes the proceeds, revealing the importance of accompanying observation of microbial properties.

1.1 Osmoadaptation of halophile microorganisms

The short-term osmoadaptation is mainly based on raising or decreasing the salt concentration in the cytoplasm by thermodynamical adjustment (**Figure 1**). This mechanism was first discovered in halophilic archaea and named *salt-in strategy*. Thereby, the organism accumulates or release counter ions, such like Cl^- and K^+, in or from the cytoplasm by proton motive force in order to maintain the ionic gradient, until the turgor is iso-osmotical with their environment. In particular, chloride was found to have specific functions for haloadaptation. However, the *salt-in strategy* requires specific adaptation of compounds included in metabolic and genetic regulation, and seems to be restricted to archaea, *Haloanaerbiales* and *Salinibacter ruber* [2, 3].

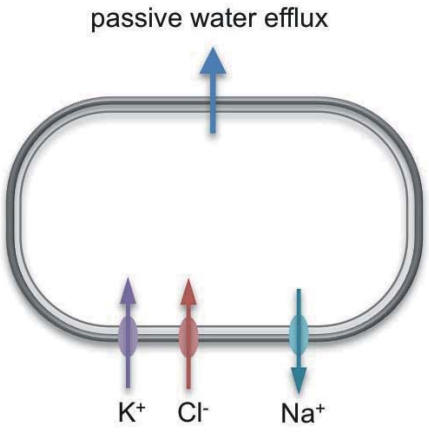

Figure 1: *Salt-in strategy*. The organism transports ions to balance the osmotic gradient and thus avoiding water efflux and desiccation.

Long-term osmoadaptation can be apportioned into evolutionary modifications and uptake or release of organic osmolytes. Since halophiles are able to live in high salinities, the cytoplasm has to deal with the similar osmolarities and internal function needs to be maintained. High ion concentrations disturb the metabolism. Hence, enzymes and proteins of halophilic microorganisms are modified to remain activity, solubility and stability at these conditions. Even, the distribution of internal amino acids is adjusted to the ionic strength to avoid precipitation and to improve the availability [1]. The surface of proteins from halophiles are generally more negative than those of non-halophiles and demonstrates higher hydrophilic properties [4]. In addition, the cell membrane, which forms a barrier between the cytoplasm and the environment, adjusts to the external salinity by alterations of membrane composition and lipid conformations [5]. However, the most investigated and discusses osmoadaptation is the organic osmolyte mechanism. The microorganisms accumulate compatible solutes inside the cells in response to osmotic stress. This step within osmoregulation is also named *salt-out strategy*, as the prior accumulation ions (*salt-in strategy*) are replaced by these osmolytes (**Figure 2**). These compounds are highly soluble in water and do not interfere with the metabolism even in high cytoplasmic concentrations [6]. The osmolytes are quasi *compatible* with the cell metabolism, which is reflected in the naming of the compound class. Compatible solutes are synthesized *de novo* or up taken directly from the environment by highly affine transporters, which is energetically preferable. Thus the *de novo* synthesis is repressed when external osmolytes are available. In general, the highly water-soluble compatible solutes fall into the chemical categories: (1) zwitterionic solutes, (2) noncharged solutes and (3) anionic solutes [3]. The wide distribution of these compounds has been reviewed currently for prokaryotes [7].

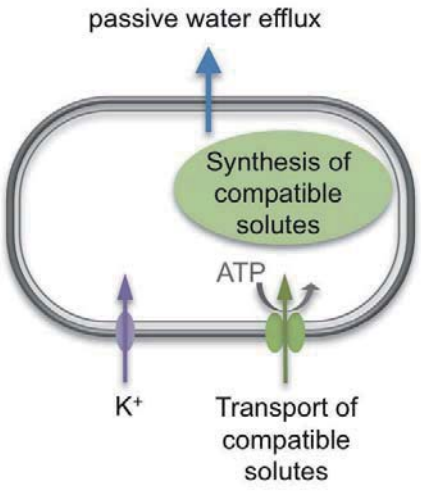

Figure 2: *Salt-out strategy*. The organism synthesizes compatible solutes *de novo* or/and transport them into the cell by high affine transporters.

Compatible solutes are able to stabilize proteins, nucleic acids and even whole cell membranes against osmotic and temperature stress, providing an advantage when living in extreme habitats. For example, protein denaturation is abated at high ionic strength. The mode of action is suggested to be thermodynamically founded and described by the

preferential exclusion model [8], which is the most popular explanation model by now. By exclusion of the compatible solutes from the hydrate envelope of the protein, the protein is forced to remain in the native structure. In addition, some compatible solutes were found to protect against UV radiation by hydration effect [9].

1.2 Cellular response on osmotic shocks

The accumulation of osmotic active compounds can disturb proliferation and viability of the microorganisms during heavy rains and floodings, as the environmental osmolarity can decrease rapidly in natural habitats. As a consequence of the osmotic gradient between turgor and environment, cell membrane rupture may occur. Therefore, microorganisms open their mechanosensitive channels to balance the osmotic pressure and avoid cell lysis (**Figure 3**). The opening of mechanosensitive channels generate large, non-selective pores within the cell membrane, which results in a non-specific efflux of intracellular compounds, such as electrolytes, compatible solutes and other metabolites [10]. When iso-osmotic conditions are achieved between turgor and environment, the mechanosensitive channels close and the organisms reassimilates on the surround condition with the object of proliferation.

The osmosensing and -regulation of these mechanosensitive channels are extensively studied on the halotolerant bacteria *Escherichia coli*, *Bacillus subtilis* and *Corynebacterium glutamicum* [10]. The several types of mechanosensitive channel were reviewed, providing an excellent overview of the structures and working principles [11, 12]. For halophiles, it is supposed that they use similar channels to maintain their cellular turgor during osmotic down-shock [13]. However, mechanosensitive channels were so far just reported for the archaeon *Haloferax volcanii* [14].

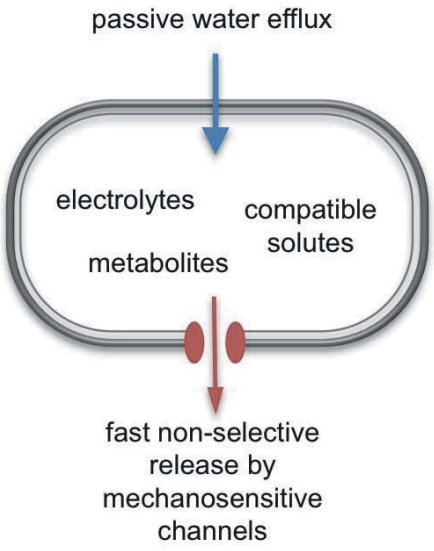

Figure 3: The cells open mechanosensitive channels to avoid cell membrane rupture and remain integrity since water pours in the cells strongly driven by osmotic gradient between environment and turgor.

Biotechnologically, this natural protection mechanism is exploited to force the halophiles to excrete their accumulated osmolytes and thus enhance downstream processing. The

bioprocess is named *bacterial milking* [13, 15]. The special natural properties of compatible solutes make them valuable and highly attractive for a lot of industrial and research applications. Within the group of compatible solutes, the amino acid derivates ectoines demonstrate extraordinary stabilization properties and thus it is already used in commercial applications and health care.

1.3 *A. haloalkaliphilus* – possibilities and chances

Among other extremophile species *Alkalibacillus haloalkaliphilus*, formerly known as *Bacillus haloalkaliphilus* and strain WN13^T, is a promising candidate for ectoine production, although it was little investigated to the present.

It was isolated from saline alkaline mud of Lake Abu Gabara, Wadi Natrun, Egypt. The cells are of rod type (0.3 - 0.5 µm x 3.9 - 8.0 µm) and able to form spherical endospores (**Figure 4**). It is motile by means of peritrichous flagella. The cell membrane is of gram-positive structure, although the gram-reaction is negative. The cell envelope contains about 79 % of saturated branched chain fatty acids and 19 % unsaturated fatty acids. The coloring (creamy white to slightly yellowish) and morphology is strongly dependent on salinity, whereas it is able to grow up until 250 g L^{-1} NaCl. When growing on salinities above 10 %, mainly ectoine is synthesized to protect against osmotic and thermal stress. Glycine betaine can be taken up and used as compatible solute too [16, 17]. Weisser and Trueper [18] even discovered that the inorganic ions Na^+, K^+ and Cl^- are used for osmoregulation. Preliminary basic studies of *A. haloalkaliphilus* revealed up to 3.6 % of the total biomass as compatible solutes, when grown on alkaline nutrient broth at about 20 % salinity [19]. This apparently low concentration might be the reason, why the organism was not further considered as ectoine producer.

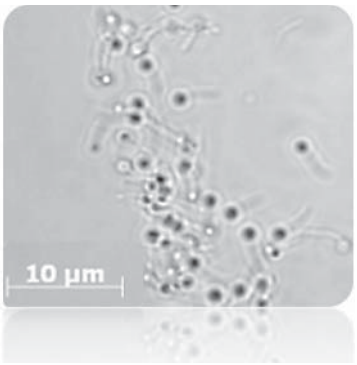

Figure 4: Endoterminally sporelated *A. haloalkaliphilus* cells grown in alkaline nutrient broth after 24 h of cultivation.

The organism is moderate halophilic, obligate alkaliphilic and strictly aerobic. Casein, gelatin and starch was reported to be hydrolyzed [17], whereas acid production only occurs from citrate and a few pentoses, such as D-xylose, D-trehalose, maltose, sucrose and cellobiose [18, 20]. The organism was reported to grow in basal mineral medium containing glucose at pH 9.7 [20]; admittedly at low optical densities, which may be resulted in an

obligate requirement of peptides [18, 19]. Good growth occurs in alkaline nutrient broth (ANB), containing the substrates soy peptone and meat extract.

Recently, the whole-genome of the allied *Alkalibacillus haloalkaliphilus* strain C-5 was sequenced, revealing deeper insights in metabolic subsystems and genes involved in stress response. The strain is known to produce a thermophilic serine protease [21].

1.4 Objectives

Given the unadvanced investigation status of the halophilic bacterium *A. haloalkaliphilus*, the chances and possibility for a holistic bioprocess on example of ectoine production

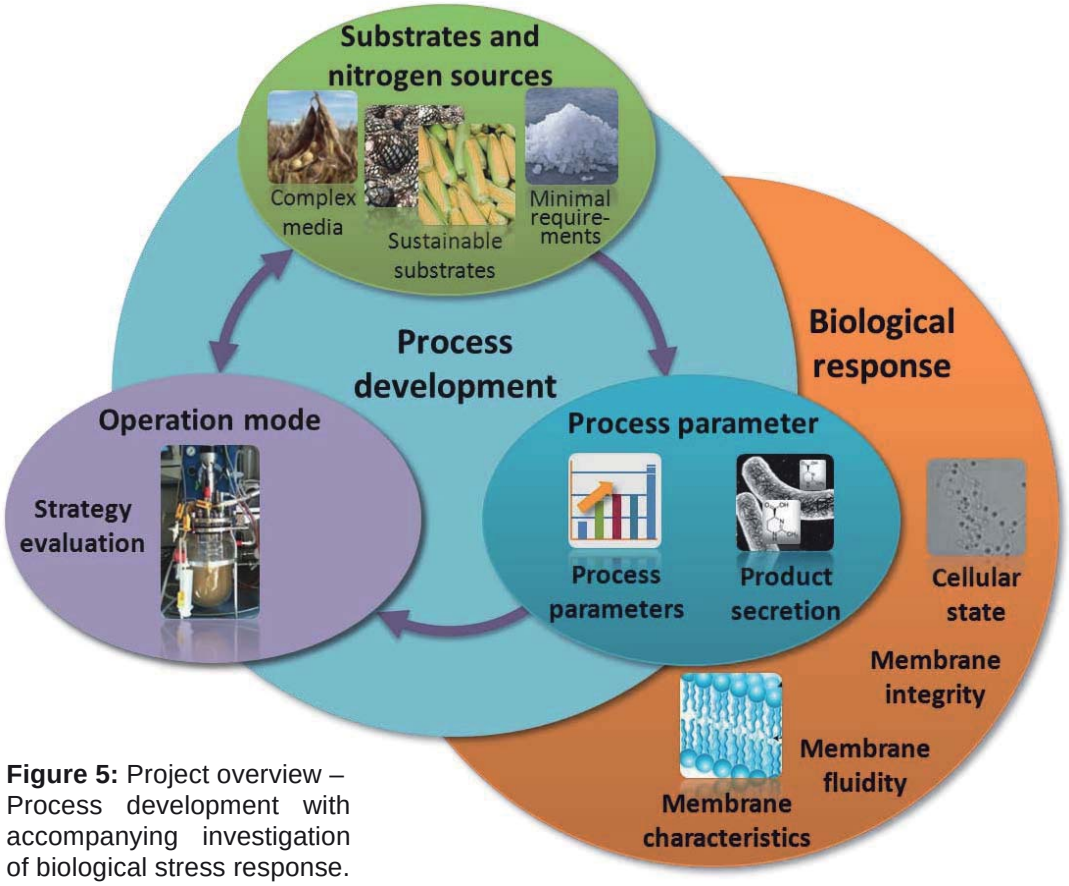

Figure 5: Project overview – Process development with accompanying investigation of biological stress response.

should be examined and the stress response characterized to improve optimization process and reveal bacterial adaptation process (**Figure 5**).

To fulfill these obligations, firstly possible and sustainable substrates shall be investigated and evaluated. Subsequently, the process key parameters salinity, pH-value and temperature shall be optimized using the power of model-based medium and process

design in order to enhance biologically relevant knowledge and the production yield. Thereby, the operation mode of a further bioprocess needs to be taken in consideration, as well as subsequent downstream processing. As ectoine is accumulated inside the cells, the cells shall be osmotic and thermal shocked, and thus forced to release the product into the environment, simplifying later downstream processing (*bacterial milking*-principle).

Accompanying measurements of the cellular physiological state during ectoine production and cell membrane properties shall reveal changes during the whole process development, giving a higher insight to the bacterial stress response. The osmotic down-shock will affect membrane integrity and fluidity. In particular, the investigation of cell membrane fluidity throughout the secretion process may reveal new aspects on biological response and support the holistic bioprocess. Since studies on the membrane properties of *A. haloalkaliphilus* are not available, comparison to another halophilic ectoine-producer provides the ability for discussion of the obtained characteristics. *Chromohalobacter salexigens* is such a well-investigated high-potential ectoine-producer.

The moderate halophile proteobacteria *C. salexigens* was firstly isolated from salines at the Netherlands Antilles. The cells are gram-negative and rod shaped (2.0 - 3.0 µ m x 0.7 - 1.0 µm). Besides several sugars, it metabolizes ethanol, acetate, citrate and other organic acids under obligate aerobic conditions [22]. To date it is considered as a kind of reference organism, besides *Halomonas elongata.* The genome is fully investigated and publicly available. Furthermore, the adaptive changes were reported during osmoadaptation within the cell membrane [23], confirming the accompany at membrane fluidity measurements.