Chapter 6

Final discussion and future perspectives

6.1. ***Physiological and biochemical behavior of*** **C. halophila** ***under salt stress***

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The availability of water is the most important prerequisite for life of any living cell, and exposure of cells to hypersaline conditions always threatens the cells with a drastic loss of water. Being part of their surrounding environment, all living organisms are exposed to the biological, chemical and physical parameters defining their habitat and, for survival, they have to be able to sense changes in environmental parameters, and react to them using various mechanisms of adaptation. Apart from the availability of nutrients, varying temperatures and pH, another frequent changing factor is the salt concentration, which is not only relevant in saline habitats, but also common in soil, in which rainfall and evaporation cause drastic changes in the environmental osmolarity.

Bioenergetic considerations that include, namely, physiological and biochemical aspects are generally neglected in surveys concerning salt stress resistance in organisms. These are very important for the understanding of the possibilities and limitations of microbial life at high salt concentrations. Detailed genetic and physiological analysis of stress responsive systems in yeasts improved greatly in the last decade, being mainly conducted in the moderately salt-tolerant yeast *S. cerevisiae*, using short periods of exposition to salt [Hohmann and Mager, 1997]. Although this yeast is used as an excellent eukaryotic model, there are still differences between this and the NCY that do not allow an immediate and straightforward translation of the available knowledge between them [Flores *et al*., 2000]. In fact yeasts metabolise very differently and this explains why they colonize different environments.

Physiological mechanisms underlying extreme salt tolerance have been the matter of intense study in Bacteria and *Archaea* elements, but is still poorly understood in yeasts. Although some studies have been made using the halotolerant yeasts *D. hansenii* and *Z. rouxii*, no coherent picture of main mechanisms involved in halotolerance exists. Much more information is needed to understand how yeast cells can survive and metabolise at high salt concentrations, especially near the limit of salt solubility.

C. halophila is a salt tolerant yeast and was isolated from soy mash, being used in the production of soy-sauce. It was selected from a salt stress resistance survey performed on 42 yeast as the most salt tolerant, being able to grow at 4.5M and 5M NaCl, respectively without and with inoculum pre-adaptation to salt [Lages *et al*., 1999]. The aim of the present study was to acquire knowledge about physiological mechanisms of long term resistance to salt stress using the extremely halotolerant yeast *C. halophila* in order to propose a metabolic model under extreme salt stress. Thus, we performed several physiological studies in cells adapted and surviving under heavy stress, *i.e*., growing on synthetic medium in the presence of salt concentrations up to 4.5 M NaCl. These included: studies on growth, osmolyte production, accumulation and transport across plasma membrane, intracellular volume and intracellular pH regulation, *in vitro* determination of enzymes activities from main metabolic pathways, as well as glycerol and mannitol pathways, measurement of fermentative and respiratory fluxes, measurement of intracellular pyridine nucleotides concentration and quantification of intracellular sodium and potassium concentrations.

As *D. hansenii* [Prista *et al*., 1997] and *P. sorbitophila* [Lages, 2000], *C. halophila* grows better in the presence of a certain amount of salt than in its absence, thus having a maximum growth rate in the presence of salt. Nevertheless, unlike some filamentous *fungi* isolated from Dead Sea and from salt fish [Andrews and Pitt, 1987; Buchalo *et al*., 1998], *C. halophila* does not depend on the presence of salt to survive. Unlike *D. hansenii* that was isolated for marine water and grows up to pH 8, *C. halophila* growth is optimum in the pH range 4.5-5.5, even in the presence of salt. This is not reflected in

intracellular pH since *C. halophila* maintains its intracellular pH in the absence and in the presence of salt up to 4M and in the external pH range 3-6 almost unaltered. *C. halophila* growth rates were only affected for salt concentrations above 2.5M NaCl, which points for its extreme halotolerant character, since other yeasts are affected at lower salt concentrations. CO_2 production and O_2 consumption rates and ethanol concentrations were determined in *C. halophila* cells in the absence and in the presence of salt. Both rates were significantly increased although at different extents in the presence of salt. *C. halophila* revealed thus to be a respiro-fermentative yeast. Ethanol increased almost linearly with salt concentration being the increase observed in CO_2 production fluxes and in the specific activity of ALD (alcohol dehydrogenase) identical (~400%). A general pattern of stimulation of enzymes from the main metabolic pathways was observed, indicating that metabolism is enhanced in the presence of salt as the enhancement of growth rates suggested.

Of the two main strategies described in literature for salt tolerance, *C. halophila*, as all known yeasts and fungi, uses the compatible solute one. Under salt stress *C. halophila* accumulates glycerol as the sole osmolyte and its accumulation correlates with salt medium concentration. In the absence of salt, mannitol is also produced, but its synthesis under salt stress is diminished and thus a role in osmoregulation is unlike. Furthermore, salt-mannitol grown cells accumulated intracellularly glycerol as the compatible solute which reinforces that postulate. Mannitol has many functions in plants namely, as compatible solute, scavenger and reserve but its function in yeasts is not clear, being suggested to act as a scavenger of toxic free radical produced by the hosts of opportunistic yeasts like *C. neoformans* [Megson *et al*., 1996]. In *C. halophila*, glycerol and mannitol pathways were tentatively assayed. Although we detected activity of enzymes involved in mannitol metabolism, due to the similarity of the assays with those used for glycerol enzymes detection, we cannot disregard the possibility that we might be measuring the activity of the same enzymes using two different substrates. This can be the case of glycerol 3-P dehydrogenase or mannitol 1-P dehydrogenase (GPD or M1PD) and glycerol or mannitol dehydrogenases (GD or MD), which catalyse the first reactions leading to glycerol or mannitol production and consumption respectively. Furthermore, glycerol and mannitol do not use the same enzyme co-factors, being some enzymes able to use both or one of them in a particular direction. The picture is aggravated by the fact that these two enzymes catalyse both the oxidation and the reduction of its substrates. Due to this ability to use more than one co-factor (which can also be to distinct isoenzymes), it may be suggested that mannitol might function as a cycle which results in a complementation of the redox couples NAD(H)/NADP(H) in a transhydrogenase-like function. This has been suggested long time ago for NADPH regeneration in fungi [Hult *et al*., 1980].

In accordance with its function as compatible solute, enzymes involved in glycerol synthesis increased its activity in the presence of salt. As *S. cerevisiae*, *C. halophila* uses the glycerol-3-P pathway for glycerol production. Interestingly GPD, a main enzyme in glycerol production, showed to use, unlike *S. cerevisiae*, both cofactors, being NADPH the one used under salt stress. This might suggest the presence of two constitutive isoenzymes and one of them may be NADPH dependent and salt responsive. This result may suggest that redox balance might be different in *C. halophila* especially under salt stress conditions. Thus, we measured the intracellular pyridine nucleotides concentration and obtained a perfect balance even under stress. In spite of this result, ethanol and acetic acid increased under stress and thus reinforces that *C. halophila* redox balance though in equilibrium under stress might be different from other yeasts, namely those that do not use a respiro-fermentative metabolism like *D. hansenii*.

Uptake of compatible solutes from the medium have been often suggested as implicated in

osmoregulation. Glycerol, glucose and mannitol transport were characterized in *C. halophila* cells and showed to be constitutively expressed. Glycerol is transported through a glycerol-H^+-symport type system, but its expression was not evaluated in salt stress conditions due to physiological limitations. Mannitol transport characterization points to an active type mechanism and is reduced substantially in salt-grown cells according with the absence of osmoregulatory function of mannitol. Glucose is transported through a facilitated diffusion type system and under sat stress follows the pattern evidenced by specific growth rate variation during growth on glucose and salt.

A common physiological/biochemical response to salt stress is the alteration of cell wall and membrane composition. These are frequently accompanied by cell volume reduction. *C. halophila* cells shocked with salt reduced its intracellular volume linearly with the external salt concentration. However, when growing actively in the presence of salt *C. halophila* cells recover its initial volume and this maintained up to 4M NaCl. Lages and collaborators (1999) suggested that the percentage reduction of intracellular volume can be correlated with the degree of tolerance to salt of each yeast. Accordingly, less tolerant yeast showed the highest reduction and the lower recover percentages.

Salts are inhibitory to yeast growth for two reasons: firstly by provoking a massive loss of intracellular water which affects the turgor pressure reducing the water available for cellular processes, and secondly by provoking a sodium specific inhibition in certain proteins. Although some fungi were characterized as halophilic, all of them extrude actively sodium in order to avoid its inhibitory effects. Thus, unlike extremely halophilic bacteria and *Archae*, it is expected that proteins from fungi be salt-sensitive. We evaluated the salt sensitivity of some enzymes in cell free extracts of *C. halophila* and verified that, similarly to what was published for the glyceraldehyde-3-phosphate dehydrogenase of *D. hansenii* [Neves *et al*., 1997], the specific activity of enzymes diminished exponentially in the presence of increasing salt concentrations. This has been already found for malate dehydrogenase and glucose-6-phosphate in the marine fungus *D. salina* [Paton and Jennings, 1988] and for isocitrate dehydrogenase in *Z. rouxii* and *S. cerevisiae* [Brown, 1976]. Furthermore, the inhibition constant was similar in *C. halophila* and in *D. hansenii*. This is not consistent with the halophilic character attributed to *D. hansenii*, namely its capacity to harbour intracellullarly higher sodium concentrations than other yeasts [Neves *et al*., 1997]. Sodium and potassium intracellular concentrations were determined in *C. halophila*. Similarly to *C. tropicalis* [García *et al*., 1997], *C. halophila* maintained low intracellular sodium concentrations and higher potassium concentrations for external salt concentrations below 2M NaCl. Above this, sodium intracellular concentration increased to approximately to 150 mM and was accompanied by a concomitant reduction in the potassium content. A strict relation between potassium intracellular concentration and the growth rate was obtained, indicating that, as postulated by several authors, potassium is a requirement for growth at least under salt stress conditions. In accordance with literature which indicates that $[Na^+]_{in}/[K^+]_{in}$ ratios above 0.5 are toxic to cells, *C. halophila* was able to maintain $[Na^+]_{in}/[K^+]_{in}$ ratios below 0.5 for external salt concentration <3M. Above 3M NaCl, $[Na^+]_{in}$ increased and because $[K^+]_{in}$ decreased the $[Na^+]_{in}/[K^+]_{in}$ ratios increased.

C. halophila growth at increasing salt concentrations showed clearly to possess three phases: (1) 0-2M; (2) 2-3M and (3) 3-4.5M. In the first phase growth rate was not affected and internal ion composition was maintained under normal parameters (Low $[Na^+]_{in}$ and high $[K^+]_{in}$). Most of the enzymes assayed also showed to increased its specific activities within this stress range. The second phase comprises a metabolic transition steep in which growth rate begins to be affected by salt and $[Na^+]_{in}$ starts to increase and $[K^+]_{in}$ to decrease. In the third phase growth is being affected

significantly evidencing progressive lower growth rates. In this phase the activity of many enzymes and $[Na^+]_{in}$ stabilized its values. The reduction of $[K^+]_{in}$ accompanied the reduction in growth rate.

Since glycerol accumulated linearly with salt up to 4M and volume and intracellular pH did not vary within this range of salt concentration, they do not account for the reduction observed in growth rates. Growth rates were not, however accompanied by similar decrease in final dry weight, which indicates that although they proliferate slower, they are metabolically effective. What seems to be the true limitation is the incapacity of *C. halophila* to maintain above 2M NaCl a higher $[K^+]_{in}$ and a low $[Na^+]_{in}$. Most probably, the extrusion mechanisms that *C. halophila* cells uses do not efficiently discriminate between Na^+ and K^+ and if is highly enhanced extrudes both ions. There are authors that defend that in order to maintain a steady ion content in the cell, when Na^+ increases, K^+ decreases. However, the sum of these two ions is not steady in *C. halophila*. Furthermore, nothing is known about the true involvement of vacuole in salt stress resistance. In *S. cerevisiae* vacuole has proved to be important in stress resistance but the exact function is still speculative. If we consider that vacuole may function as a sodium storage compartment, then our results will have only one explanation, that vacuole overflow for external salt concentrations above 2M.

It has been suggested that yeasts able to maintain low $[Na^+]_{in}$ may be named as *excluders*, while the ones withstanding higher $[Na^+]_{in}$ were *includers* [Ramos, 1999]. It is clear that *C. halophila* excludes, at least up to 2M NaCl, efficiently Na^+. However a question remains: are there true *includers* among yeasts? Consistently with the results here presented it can be suggested that ion fluxes, and not only sodium extrusion, are fundamental in yeast tolerance and its efficiency seems to be one important factor discriminating the tolerance degree among yeasts. This is supported by the fact that *S. cerevisiae* is moderately tolerant and its $[K^+]_{in}$ decreases with any salt concentration in the medium being accompanied by an increase in $[Na^+]_{in}$ [García *et al*., 1997].

Taking all the results obtained into consideration, we think that they provide substantial evidence that *C. halophila* is a good model in representing the limits of extreme halotolerance in yeasts and supports the idea that even extremely salt tolerant yeast should not be considered true halophilic microorganisms.

6.2. Future perspectives

Our understanding of the molecular physiology of the osmotic stress response in yeasts has undergone considerable improvements during the last decade. The understanding of the metabolic complexity has mainly originated from genetic analysis of mutants and expression analysis by proteomics. Additionally, the advent of DNA microarray technology and its use in large-scale transcript analysis during saline stress conditions enlarged greatly the knowledge on stress response. Nevertheless, the studies presented in this thesis are unique in the long-term response to heavy salt stress in yeasts, since most of the studies published refer to short periods of exposition to salt and recently are focusing essentially in genetic molecular analysis.

C. halophila proved to be and extremely halotolerant yeast of unquestionable value in the acquisition of salt stress knowledge. It is more tolerant to salt than *D. hansenii* and according to literature behaves differently in physiological and biochemical terms. This is coherent with both the different degree of salt tolerance exhibited by those yeasts, but also with the different places from which they have been isolated and the intrinsic metabolism of each yeast.

Considering the state of art in what refers to salt stress response in yeasts and taking into

consideration all the results obtained, we consider that the studies performed on *C. halophila* salt stress response open many possibilities. These may include:

- Identification of the role of constitutive mannitol production in yeasts, namely its possible relation with oxidative stress in the infection of opportunistic pathogens or its relation with alternative redox balance systems in the absence and in the presence of salt.

- Cloning, isolation and characterization of ion transport genes implicated in salt stress tolerance, with the comparative study with ion transport systems characterized in other yeasts and its functional expression.

- Cloning, purification and characterization enzymes from glycerol and mannitol pathways. Functional expression and its regulation at transcriptional, post transcriptional, translational and biochemical level.

- Cloning and heterologous expression of *C. halophila* glycerol 3-P dehydrogenase in order to (1) confirm its co-factor versatility and (2) to verify the possibility to help *S. cerevisiae* to evercome redox imbalances under different metabolic regulation conditions. This would increase the biotechnological potential of this yeast and open new perspectives in industrial applications.

- Finally, the study of the promoters from the enzymes studied in *C. halophila* may reveal to be very interesting since they are most probably sensitive to the induction by salt-stress in a much more direct and strong manner as the cases known from *S. cerevisiae*.