Chapter 1

General Introduction

1.1. Yeasts and Non-Conventional Yeasts (NCY)

Yeasts are an attractive group of lower eukaryotic microorganisms, some of which are used in several industrial processes that include wine making, brewing, baking and the production of a variety of biochemical compounds [Boekhout and Kurtzman, 1996; Domínguez *et al.*, 1998; Boekhout and Robert, 2003]. Yeasts also cause spoilage of foods and beverages and are of medical importance [Wolf, 1996; Boekhout and Robert, 2003]. *Saccharomyces cerevisiae* has usually been the yeast of choice, but an increasing number of alternative non-*Saccharomyces* yeasts are nowadays under study [Wolf, 1996; Flores *et al.,* 2000; Wolf *et al*., 2003]. Some of them exhibit certain favourable characteristics offering significant advantages over traditional baker's yeast [Flores *et al.,* 2000]. In fact, the so-called non-conventional yeasts have been used to produce polysaccharides, food ingredients, feed additives, fuels, chemicals and more recently pharmaceuticals [Wolf, 1996]. Furthermore they are also used for heterologous gene expression of proteins with considerable pharmaceutical, therapeutic and commercial interest [Domínguez *et al*., 1998]. *Arxula adeninivorans* [Wartmann *et al*., 1995; Kunze and Kunze, 1996, Wartmann and Kunze, 2003], *Candida maltosa* [Mauersberger *et al*., 1996; Arie *et al*., 2003], *Debaryomyces hansenii* [Adler *et al*., 1985; Ochoa *et al*., 2003; Thomé, 2003; Ramos and Almagro, 2003], *Hansenula polymorpha* [Hansen and Hollenberg 1996; Gancedo *et al*., 2003], *Hortae werneckii* [Petrovic *et al*., 1999; Gunde-Cimerman *et al*., 2000; Petrovic and Plemenitas 2003], *Kluyveromyces lactis* [Wésolowski-Louvel *et al*., 1996; Breunig *et al*., 2000; Fichtner *et al*., 2003], *Pichia stipitis* [Melake *et al*., 1996; Passoth *et al*., 2003], *Pichia pastoris* [Gould *et al*., 1992; Streekrishna and Kropp, 1996], *Pichia guilliermondii* [Sibirny, 1996], *Schwanniomyces occidentalis* [Wilson *et al*., 1982; Calleja *et al*., 2003], *Yarrowia lipolytica* [Barth and Gaillardin, 1996; Mauersberger *et al*., 2003], *Zygosaccharomyces bailii* [Thomas and Davenport, 1985; Warth, 1991; Rodrigues *et al*., 2003], *Zygosaccharomyces rouxii* [Restaino and Lenovich, 1982; Soto *et al*., 2003], and also the human opportunist pathogens like *Candida albicans* [Dupont, 1995] and *Cryptococcus neoformans* [Manfredi *et al.,* 2002], are yeast species already well established has being important in some area of research or biotechnology [Wolf, 1996; Domínguez *et al*., 1998; Wolf *et al*., 2003]. In spite of their importance, basic physiological and biochemical knowledge on enzymes, fundamental pathways and gene regulation is relatively scarce in these yeasts [Flores *et al.,* 2000]. However, the use of this type of yeasts is being aided by the continued advances in bioinformatics, metabolic and genetic engineering, that greatly enhance our understanding of yeast metabolism, genetics and biochemistry [Charles, 2001]. Thus considering, the term "non-conventional yeasts" is gradually loosing its significance and usefulness, since these yeasts are more and more gaining importance in fundamental and applied studies and processes [ISSY, International Specialized Symposium on Yeasts, 2001], as soon as more information about its properties is being acquired [ISSY, 2001].

1.2. Eukaryotic models

There are several reasons why the budding yeast *S. cerevisiae* has been selected through years as an excellent model system for eukaryotes. This yeast is the most ancient documented organism to be used in everyday life. It is listed as a GRAS (**G**enerally **R**egarded **a**s **S**afe), being a convenient host for the production of foreigner (heterologous) proteins. The genome of *S. cerevisiae* is completely sequenced since 1996, which reflects its importance as a model organism in the research on eukaryotic cellular physiology. This, together with the diverse and efficient yeast genetic tools available, the easy transformation, the fast cultivation with few requirements, the efficient expression of heterologous genes and the many features shared with multicellular organisms, made *S. cerevisiae* a widespread

eukaryotic model. However, often other organisms are being used as preference models. For instance, *Drosophila melanogaster* is a better model than *S. cerevisiae* for understanding the molecular basis of human diseases, as well as embryogenesis and tissue differenciation, since it contains more ortholog genes associated with humans disease genes than yeasts [Chervitz *et al.,* 1998; Rubin *et al.,* 2000]. The fission yeast *Schizosaccharomyces pombe* is actually the most used species in cell cycle and morphogenesis research being proposed several times as a eukaryotic model in these fields. Wolf (1996) in his book on "nonconventional yeasts", considered that all yeasts described were nonconventional with exception of *S. pombe* and *S. cerevisiae*. Some studies on fission yeast complement very well those of the budding yeast, because some mechanisms are similar, though distinct, and many times have been claimed to be more related to those of the higher animals [Glick, 1996]. Now that its genome has also been sequenced [Wood *et al.,* 2002], *S. pombe* is ready to definitely assume its place near the five eukaryotic models whose genomes have been already sequenced or are approaching its end, which are the budding yeast *S. cerevisiae*, the nematode worm *Caenorhabditis elegans*, the fruit fly *D. melanogaster*, mustard weed *Arabidopsis thaliana* and the human *Homo sapiens* [Yanagida, 2002]. It seems that other eukaryotic models will emerge rapidly in the near future, as an obvious consequence of the rapid availability of more sequenced genomes, together with the development of bioinformatic and genetic tools and also specific and faster analysis equipments. There is no doubt that much of what we learn using microorganisms as model systems will have an important influence on our understanding of the mechanisms by which mammals and other higher eukaryotes cope with a permanent changing environment.

1.3. Studies on salt stress in yeasts and their applications

Studies on stress are not only interesting on a scientific basis but also have medical and economical relevance [Ruis *et al.*, 1995]. Stress factors contribute to cellular aging, are involved in the etiology of cancer and in immune surveillance and play a role when tissues are exposed to hypoxia, *e.g.*, in the ischemic heart and brain [Young and Elliot, 1989; Welch, 1993; Ames *et al.*, 1993]. On the other hand, resistance of higher plants to stress conditions exerted by salt, heat or drought is particularly important in agriculture [Boyer, 1982].

About 800 species of yeasts are presently known [Barnett *et al.*, 2000], and several play significant roles in the food, brewing, wine and beverage industry. Yeast can be either beneficial or detrimental [Boekhout and Robert, 2003], as mentioned above, being in many cases the relantionship between these two aspects a fragile balance, which depends on the interplay between various biotic and abiotic factors. Stress in yeast cells cannot always be avoided in industrial applications where they are used, and is often a constant environmental factor for yeast cells that spoil food or their ingredients or infect human beings. Yeast cells may face simultaneously different types of stress, being the most common provoked by osmolarity, heat, starvation and oxidants. Only the osmotic stress will be approached in the scope of this thesis.

The study of the physiological and molecular mechanisms underlying yeast osmoregulation and osmoadaptation relate to the importance of these aspects in biotechnology and in agriculture. For instance, improving crop resistance to osmotic stresses is a long-standing goal of agricultural biotechnology [Nuccio *et al.*, 1999]. In fact, the most important factors limiting plant productivity are environmental stresses, of which salinity and drought are the most serious [Serrano, 1994]. Salinity affects more than 40% of irrigated land, specially the most productive areas of the world. This is due

to the cumulative effects over decades or centuries of adding water with some dissolved salt to the soil of arid regions. Thus, the understanding of ion homeostasis mechanisms and the creation of salt tolerant yeasts [Attfield, 1997] and plants [Nuccio *et al.*, 1999; Serrano and Rodríguez-Navarro, 2001] by metabolic engineering is a very important goal of salt stress research. On the other hand, the need to improve the performance of yeast strains under industrial conditions [Remize *et al.*, 1999; Randez-Gil *et al.*, 1999; Pretorius, 2000] and food preservation methods [Troler *et al.*, 1978; Hahn-Hägerdal, 1986; Fleet, 1992; Wind and Restaino, 1995; Gutierrez *et al.*, 1995], which are often associated with rapid alterations in water activity and specially with high osmolarity, requiring a better understanding of the impact of low water activity on yeasts cells, contribute and justify the raising of yeast osmoregulation knowledge in the last years. Yeasts have revealed through years that the information of their genes may be successefully transferred to other microorganisms, plants and other higher eukaryotes, resulting in the improvement of resistance capabilities [Domínguez *et al.*, 1998]. Thus, heterologous expression of genes associated with salt stress resistance remains one of the most important goals in salt stress research. However, the mechanisms underlying long-term resistance to high salt concentrations are yet poorly understood.

References on extremely salt resistant yeasts are very scarce in literature. *S. cerevisiae,* despite being only slightly tolerant to salt, has been so far the most common yeast used to study salt stress response [Hohmann, 1997, 2002]. Besides *S. cerevisiae*, the most studied yeast in the salt stress field is the halotolerant yeast *D. hansenii* [Adler and Gustafsson, 1980; Adler *et al.*, 1985; Tunblad-Johanson *et al.*, 1987; Gustafsson and Larsson, 1987; André *et al.*, 1988; Burke and Jennings, 1990; Lucas *et al.*, 1990; Larsson *et al.*, 1990; Jovall *et al.*, 1990; Larsson and Gustafsson, 1993; Prista and Madeira-Lopes, 1995; Neves *et al.*, 1997; Prista *et al.*, 1997; Thomé-Ortiz *et al.*, 1998; Ramos, 1999; Thomé and Trench, 1999; Almagro *et al.*, 2000, 2001, Almagro and Ramos, 2003]. Furthermore, a few isolated studies, concerning salt stress, have been made in other yeasts like: *A. adeninivorans* [Yang *et al.*, 2000], *Candida tropicalis* [García *et al*, 1997; Ali *et al.*, 2001], *Candida membranefaciens* [Khaware *et al.*, 1995], *H. werneckii* [Gunde-Cimmerman *et al.*, 2000; Petrovic *et al.*, 1999, 2002; Turk and Plemenitas, 2002], *Pichia sorbitophila* [Lages and Lucas, 1995; Bañuelos *et al.*, 2002; Maresova and Sychrova, 2003], *S. pombe* [Rothe and Höfer, 1994; Hahnenberger *et al.*, 1996; Balcells *et al.*, 1999], *S. occidentalis* [Bañuelos *et al.*, 1998], *Y. lipolytica* [Andreischeva *et al.*, 1999] and *Z. rouxii* [Koh, 1975; Edgley and Brown, 1983; Hamada *et al.*, 1984; Steinkraus *et al.*, 1985; Ushio *et al.*, 1991; Hosono, 1992; van Zyl *et al.*, 1990, 1991, 1993; Nishi and Yagi, 1992, 1993, 1995; Watanabe *et al.*, 1991a,b, 1993b, 1995, 1999; Ohshiro and Yagi, 1996; Iwaki *et al.*, 1998, 2001; Jansen *et al.*, 2003]. In spite of that, there is almost no substantial knowledge on basic areas of physiology, biochemistry and even genetics related with salt stress in these and other yeasts [Flores *et al.*, 2000]. The need to acquire, compare and apply new intrinsic aspects of extreme halotolerance, suggests the study of more halotolerant yeasts, since they represent potentially good models to unveil some of the physiological and molecular mechanisms underlying yeast long-term extreme tolerance to salt, which can not be approached in less tolerant yeasts such as *S. cerevisiae*.

1.4. The yeast *Candida halophila* (syn. *versatilis*) CBS 4019

Candida halophila was isolated from soy mash [Onishi, 1957]. This corresponds to an intermediate phase of soy sauce production. Soy sauce is made from a mash of soy beans and malted grain that is left to ferment approximately 3-4 days. It is then combined with high salt brine and matured for several months [Hanya and Nakadai, 2003]. Soy mash has a high salt content (about 17% (w/v)), being *C. halophila* added frequently to enhance the production of phenolic compounds, which are important to the quality of the soy sauce [Hanya and Nakadai, 2003]. Due to its high salt tolerance and

its ability to withstand the presence of some ethanol content and a moderate low pH, *C. halophila* predominate in the later stages of the soy fermentation process [Hanya and Nakadai, 2003]. According to Suezawa (1995), *C. halophila* produces phenolic compounds like 4-ethylguaiacol, which give an important and characteristic flavour to soy sauce. Thus *C. halophila* is actually used in a continuous reactor for flavour production in development of soy-sauce processes [van der Sluis *et al.*, 2000, 2001]. On the other hand, Lages and collaborators (1999) performed a salt stress resistance survey in which *C. halophila* was the only yeast able to grow in the presence of sodium chloride concentrations as high as 4.5M or 5M when adapted to grow in the presence of salt. Considering the reasoning above, the implication of *C. halophila* in the production of fermented foods and its extreme salt tolerance make this yeast a potentially good microorganism to unveil the physiological mechanisms underlying extreme salt-stress tolerance. For these reasons we selected *C. halophila* as the biological material for the study of the long-term physiological responses to salt stress provoked by NaCl from 0.5 M to 4.5 M.

1.5. Stress definition, osmotic stress, salt stress and other concepts

What factors cause stress to cells? Stress is a condition dificult to define, because it depends on the definition of what is a non-stressful condition and on what is considered the cause of stress. At first glance, we may say that the conditions allowing the fastest growth will be optimal, for a particular cell, and hence non stressful, although this implies that most of the known conditions for growth will be stressful [Eriksson, 1996] and make the definition meaningless [Norbeck, 1997]. Optimal growth has been also regarded as the one that allows the cell to maintain an optimal internal composition, *i.e.*, homeostasis. Stress is usually viewed as the lack of homeostasis. Since yeasts are ubiquously found living in many diverse and variable environments, presenting different growth performances, it can be expected that, due to a natural actively changing environment, yeast should possess all the mechanisms that a eukaryotic cell requires to respond and to adapt to changes in order to maintain all the time its homeostasis, otherwise it would not grow or even stay alive. This means that cells are almost permanently under stress or better under environmental changes [Hohmann, 1997]. The difficulty lays in separating these environmental changes from those we call stress situations. Considering that the resistance of each microorganism to changes in their environment and its intrinsic biological needs differ considerably, is it possible to define stress in general? There is not a clear answer for this question and the stress term has to be explicited for each particular case. In one level, the term refers to those situations that restrict or prevent growth and reproduction of a great variety of microorganisms. But in another level, a stressful environment is one that differs significantly from the previous culture conditions. Whatever the stress definition, there are microorganisms living in extreme environmental conditions which are termed extremophiles [Horikoshi and Grant, 1998]. In the scope of this thesis we will consider that stress is being applied to a cell any time its physical or chemical properties are changed in result of the alteration of one single parameter in environmental surrounding, like for instance an increase or decrease of pH, temperature oscillation, variation in the medium composition or in the availability of nutrients, the presence of harmful substances like salt and so on. On the other hand, homeostasis will be referred as the internal equilibrium established between the organism and its surrounding medium, which allows growth. Homeostasis is, according to this definition, strictly related to a specific organism in a specific environment, meaning it will vary following the cells needs.

All biochemical reactions involve water and when the availability of free water is too low, the life process stops [Gervais *et al*., 1996; Eriksson, 1996]. Water plays a central role in life as a solvent for most of the components of cells, thus influencing the structure of proteins, nucleic acids, membranes and the size and shape of the cell [Wiggins, 1990]. Hence, it appears to be essential for all types of cells to maintain and to control their water content within certain limits, since this is determining the normal functioning of the cells general metabolism. The thermodynamic state of water can be described by different but strictly related physico-chemical parameters like: osmotic pressure, osmolarity, water potential, chemical potential of water, osmotic potential and water activity [Blomberg and Adler, 1992]. Water activity (a_w) is the parameter most used by microbiologists [Hahn-Hägerdal, 1986] and represents the thermodynamically available water for biochemical reactions.

When the cell surroundings is submitted to a decrease in water activity, and hence an increase in solutes concentration, we say that the cell is being faced with osmotic stress. Osmotic stress can be provoked by increasing the concentration of various substances in the medium like, for example, sugars, polyols or ions. Salt stress results from the presence of high salt concentrations and is distinguished from osmotic stress in the base of its effects on the cell, since it induces not only osmotic problems concerning the chemical species concentration but also toxic effects related with the ions properties [Serrano, 1996]. Considering all the concepts above we stress two important ones commonly used in what regards cells response to low a_w stress which are osmoregulation and osmoadaptation. According to Brown and collaborators (1986), osmoregulation aims "the maintenance of turgor pressure and/or cell volume within limits necessary for growth and multiplication of an organism". This definition was later completed by Wood (1999) that termed it as "the physiological processes that mitigates passive adjustments in cell structure caused by changes in the extracellular osmolarity". This concept was used more often in the past by plant and animal physiologists than by microbiologists [Brown, 1978]. Nowadays it is widely used, though sometimes missused [Reed, 1984]. Osmoadaptation is part of cellular osmoregulation, which plays an important yet not fully appreciated role in cell growth and morphogenesis [Hohmann, 2002] and describes both the physiological and genetic manifestations of adaptation to low and high water environments [Galinski, 1995]. Thus, the nature and extent of osmoadaption processes determines the intrinsic limits of environmental tolerance that allows a specific type of cell to survive and proliferate.

Cells exposed to an increase in salt medium concentration suffer a rapid outflow of water [Morris *et al*., 1986]. This process is called osmosis and consists in the movement of solutes, usually through a membrane, from a compartment with higher concentration to a compartment with lower concentration [Blomberg and Adler, 1992]. In order to grow, cells must maintain a positive turgor pressure, which is by definition the hydrostatic pressure difference that balances the osmotic pressure difference between cell interior and exterior [Wood, 1999]. Turgor pressure is exerted from the cytoplasm to the cell wall being responsible for cell expansion during growth. An important consequence of the water outflow is the lack of an internal cellular pressure, a prerequisite for an increase in cell volume and start of cellular division [Philips and Herskowitz, 1997].

1.6. Effects of salt in water structure and in water availability

The characteristic tetrahedral structure of water is known to be disrupted by changes in pressure and temperature [Postorino *et al*., 1993], being suggested that ions in solution might have a similar disturbing effect [Tromp *et al*., 1992]. Ions induce a change in water structure equivalent to the

application of high pressures, and the degree of the effect is ion-specific [Leberman and Soper, 1995]. High concentrations of sodium ions are very effective in protein precipitation (salting-out phenomenon). Since ions, on average, bind more water than proteins, the competition between dissolved salt and dissolved protein results in a loss of protein solubilization. In comparison, and for the same reason, salts lower the water activity more than sugars at the same concentration [Hahn-Hägerdal, 1986; Tokuoka and Ishitani, 1991]. How does the presence of salt change water availability? According to Wiggins (1990), the water inside biological systems has different properties from place to place, *i.e.*, water is more or less reactive (dense) depending on the concentration and type of molecules present. Dense water has a low viscosity and is extremely reactive because many water molecules are unbonded, having free OH groups, which are the reactive centers of water molecules. Low-density water has higher viscosity and is inert because water molecules are strongly bonded between them. Water equilibrates by increasing in density where the concentration of solutes is high and decreasing in density where the concentration of solutes is low [Wiggins, 1990]. Biologically, the most important secondary effects of increases or decreases in the density of water are changes in its ability to dissolve solutes. Water dissolves ions easily, because the polar molecules orient themselves round ions, and thus partially neutralize their charges. In order to acquire their stabilizing sheath of water molecules these need to form as many hydrogen bonds with one another as possible. Thus, ions compete with water molecules and clearlly it is easier to be hydrated by dense, weakly bonded, reactive water, then by low-density, strongly bonded, inert water. The more water molecules a particular ion needs to stabilize in aqueous solution, the more it will prefer dense water. This way each ion has a specific preference for each water type that is related with its size and charge. Small highly hydrated cations tend to accumulate in a more dense phase, while larger, singly charged cations *prefer* the less dense phase. Na^+ and K^+, which behave very similarly in normal aqueous solutions, partition in opposite manner. Na^+ and K^+ accumulate preferentially in high- and low-density water, respectively [Wiggins, 1990]. Wiggins (1988) suggested that this was the mechanism that determined their remarkable biological specificity. In a simplistic way, it seems that upon an osmotic upshift cells try to avoid the loss of water and the existence of intracellularly undesirable physical structure of water. The selective accumulation of K^+ and the production and retention of solutes other than ions, seem to be cellular strategies to achieve that goal. These strategies are supported by the natural tendency to equilibrate charges as well as external osmotic pressure. In fact, most of the vital components of the cell are charged such as, for example, proteins and DNA, and their stability and catalytic activity depends on the availability of water and intracellular ions. In addition to the osmotic effect caused by a_w reduction due to the massive presence of ions, at high concentrations, sodium is toxic for most enzymes since it also causes a change on the hydrophobic-electrostatic balance between the forces maintaining protein structure [Serrano, 1996].

1.7. Salt stress response in yeasts

As inhabitants of natural and artificial aqueous environments [Spencer and Spencer, 1997], yeasts can survive dramatic changes in extracellular water activity. Survival of yeast cells is both dependent on their ability to sense alterations in the environment and to appropriately respond to a new situation [Mager and Hohmann, 1997]. Therefore, stress control plays an important role in the regulation of the yeast cellular growth potential. Under extreme environmental circumstances, organisms generally undergo a rapid change in their cellular machinery, modulating metabolic pathways and changing gene

expression [Causton *et al.*, 2001]. The molecular and physiological mechanisms induced upon exposure of cells to adverse conditions are commonly designated stress responses [Mager and Hohmann, 1997]. These response mechanisms aim to protect cells against detrimental effects and to repair any molecular damage. Consequently the cellular processes and the metabolism are adjusted to a new status. Indeed, the survival mechanisms should be an intrinsic propertie of the cell allowing a rapid recover under harsh conditions [Hohmann, 2002].

Yeast cells can be subjected to different types of stress [Hohmann and Mager, 1997]. Stress conditions are sensed by cells and trigger both specific and general molecular responses according to the organism and their resistance level [Gasch *et al.*, 2000]. Although each type of stress has specific responses they form a small, fairly unstudied part of the global cellular response elicited by stress [Causton *et al.*, 2001, Hohmann, 2002]. Yeasts exposed to a mild stress develop tolerance not only to higher doses of the same stress, but also to stress caused by other agents. This phenomenon, known as cross-protection, suggests the existence of an integrating mechanism that senses and responds to different forms of stress nowadays termed as General Stress Response [Trollmo *et al.*, 1988, Lewis *et al.*, 1995; Estruch, 2000; Causton *et al.*, 2001; Hohmann, 2002]. The overlap between the various stress responses can contribute to the phenomenon of cross protection but, essentially points to the idea of the existence of one central molecular mechanism which can be activated by various factors and, upon activation, protect cells against a number of conditions threatening their survival, *i.e.*, a vital basic response essentially involved in cell homeostasis control [Ruis and Schüller, 1995; Siderius *et al.*, 2000]. Furthermore, osmotic alterations with implications in cell division have been reported [Philips and Herskowitz, 1997; Alonso-Monge *et al.*, 2001], emphasizing either the importance of osmotic control in the life of organisms, namely their capacity to grow and proliferate, and the strict relations in the regulation of different metabolic aspects of the cells [Rep *et al.*, 1999; Yenush *et al.*, 2002].

1.7.1. *Microbial strategies for survival under salt stress*

Modulation of cytoplasmic solvent composition is the only cellular response that is known to reverse the growth-inhibitory effects of osmotic shifts in prokaryotes and eukaryotes [Oren, 1999]. Additionally, two fundamentally different strategies exist within the microbial world that enable microorganisms to cope with the osmotic stress inherent to the presence of high salt concentrations (1) the "salt-in" strategy, in which the cells maintain high intracellular salt concentration similar to the external concentration and (2) the "compatible-solute" strategy, in which the cells maintain low salt concentration in the cytoplasm and accumulate low molecular weight solutes commonly termed osmolytes or compatible solutes [Oren, 1999]. The salt-in strategy is characteristic of the aerobic extremely halophilic *Archaea* of the order *Halobacteriales* [Bayley and Morton, 1978; Kushner, 1985; Lanyi, 1974], the anaerobic halophilic bacteria of the order *Haloanaerobiales* [Oren, 1986; 1993], and the moderately halophilic aerobic bacteria [Ventosa *et al.*, 1998]. In cells using this strategy for salt stress adaptation, all enzymes and structural cell components have to be adapted to the presence of high salt concentrations in order to ensure the proper functioning of the intracellular machinery [Oren, 1999]. Enzymes are thus salt tolerant both in the aerobic *Archaea* [Lanyi, 1974] and the anaerobic bacteria of the order *Haloanaerobiales* [Eisenberg, 1995], being strictly confined to organisms naturally living in environments of high osmolarity.

In most other halophilic and halotolerant microorganisms, the osmotic balance is provided by small organic molecules that are either synthesized by the cells or taken up from the medium when

available [Brown, 1976, 1978]. The compatible-solute strategy has as its hallmarks (1) a minimal requirement for genetic change (the so-called "genetic simplicity" [Yancey *et al.*, 1982]), since it does not involve the need for specially adapted proteins, and (2) a high degree of flexibility in allowing organisms to adapt to significant fluctuations in external osmolarity.

The only strategy, so far, reported in yeasts is the compatible solute one. In order to restore a metabolically functional internal milieu and a turgor pressure consistent with growth, yeasts accumulate osmotically active compounds, termed osmolytes or compatible solutes [Brown and Simpson, 1972; Brown, 1978; Yancey *et al.*, 1982; Jennings and Burke, 1990a]. Osmolytes are by definition compounds that can be accumulated by endogenous production or by uptake from the medium to high concentrations without giving rise to appreciable enzyme inhibition or inactivation [Brown, 1976, 1978, 1990]. Compatible solutes, in addition to their role as osmotic balancers, also function as effective stabilizers of enzyme function providing protection at high concentrations against, salinity, high temperature, freeze-thaw treatment and even drying [Brown, 1976, 1990]. Osmolytes used by different organisms are mainly amino acids, amino acid derivatives, potassium ions and polyalcohols, like glycerol, mannitol, arabinitol and erythritol, but the list is steadily growing [Brown and Simpson, 1972; Brown, 1978; Yancey *et al.*, 1982; Jennings and Burke, 1990; Galinski, 1995]. There is a hierarchy of preference among the solutes that accumulate in response to an osmotic upshift. For example, in bacteria, particular zwitterionic organic co-solvents such as ectoine and glycine betaine perform this role more often than inorganic solutes such as K^+ [Wood, 1999]. On the other hand, the compatible solute most used by yeasts when facing salt stress is the small polyol glycerol, which the cells produce and accumulate [Brown and Simpson, 1972; Brown, 1978; Yancey *et al.*, 1982; Jennings and Burke, 1990]. Among eukaryotes, glycerol is the osmolyte preferred for osmoregulation. This preference lies in the glycerol structure and physical properties. Its solubility in molar terms exceeds that of all other solutes, since it is highly miscible with water [Oren, 1999]. Moreover, even at extremely high concentrations (4M and more) it still supports excellent activity of intracellular enzymes like for instance in the algae *Dunaliella parva* [Ben-Amotz and Avron, 1973]. Osmoregulation of uptake, efflux, biosynthesis, retention and/or catabolism is required to modulate the cytoplasmic levels of compatible solutes [Blomberg and Adler, 1992; Wood, 1999].

1.7.2. *Phases of the osmotic response*

Yeasts have evolved to be extremely responsive and adaptative to environmental changes and when under conditions that are not physiologically ideal, they exhibit a complex array of stress responses [Hohmann, 1997a]. In fact, an increase in external osmolarity is stressful to yeasts, and these respond both passively and actively inducing many physiological changes.

Since cell permeability to water is high, imbalances between turgor pressure and osmotic pressure across cell wall provokes fast physical changes [Morris *et al*., 1986; Meikle *et al*., 1988; Albertyn *et al*., 1994b]. On the other hand, changes in cell structure, organization and composition that result from transmembrane water flux trigger and are modulated by physiological responses, which aim lead the cell to homeostasis. Yeast response to a salt shock may be essentially described in three phases [van Wuytswinkel *et al.*, 2000; Mager and Siderius, 2002] (1) dehydration (loss of some cell water) with perception of an external signal usually, through the action of osmosensors; (2) intracellular transmission of the signal sensed through signal transduction pathways, leading to the regulation of gene expression which consequently triggers physiological and biochemical changes in order to adjust cytoplasm

composition and to enable the recover of cell volume (adaptation process) and, (3) a state of balanced growth results from the cellular remodelling achieved during the previous phases. Many of the cellular responses triggered by osmotic stimuli occur, most probably, simultaneously. In fact, there is still limited experimental evidence in the time course of osmoregulatory processes and its relation with the maximum salt resistance of each strain [Hohmann, 2002]. It seems obvious that the time course events depend on the severity of the salt shock, *i.e.*, the time window being smaller after a mild shock and progressively longer after a more severe shock [Yale and Bohnert, 2001; Hohmann, 2002]. Two types of responses in cells response to stress have been distinguished: the early or immediate responses and the late or delayed responses [Ruis and Schüller, 1995]. Immediate or early responses (*e.g.* the action of some protein kinases or the trehalose metabolism) appear to have two functions: they provide a minimal protection against sudden stress, and they initiate delayed late responses, *e.g.*, the synthesis of heat shock proteins and enzymes scavenging toxic oxygen radicals [Ruis and Schüller, 1995]. Delayed responses will protect cells more permanently and effectively by allowing an adaptation to persistent stress [Ruis and Schüller, 1995; Hohmann, 2002]. Knowledge on time course of salt stress response is scarce and, although some studies concerning the phosphorylation of HOG pathway components like Hog1p [Rep *et al*., 1999, 2000], and polyol accumulation exist [Albertyn *et al*., 1994a], cell response needs to be further investigated in what concerns long time scale response. Moreover, the time required to respond osmotically is most certainly different from yeast to yeast, being probably correlated with their intrinsic salt resistance. In Table 1.1 it was attempted to list the main structural and physiological events that follow a salt shock in yeast cells.

Table 1.1. Phases of osmotic stress response in the yeast *S. cerevisiae*. Structural and physiological responses triggered by salt shock. The time presented for each phase must be viewed as an estimation since real time is not yet known for most of the process occurring in the phases represented. Furthermore each phase will depend not only on the severity of the salt shock [Yale and Bohnert, 2001], but, most probably, also on the resistance class of each yeast.

Phase/Time	Structural and physiological changes
I (≈1 to 2 minutes)	Cell shrinks (dehydrates) [Morris *et al*., 1986; Meikle *et al*., 1988; Albertyn *et al*., 1994b]. Under severe salt stress the cytoplasm can collapse (plasmolysis) and death will occur. The water loss is a rapid event, usually occurs in less than a minute [Albertyn *et al*., 1994a]. As a consequence, cytoplasmic a_w decreases. ΔpH will probably be transiently altered. The physical state of cell wall and membrane change and most membrane transport systems cease to function [Beney and Gervais, 2001; Yenush *et al*., 2002]. Cell cycle arrests due to the reduction of the expression of the genes encoding ribosomal proteins and proteins involved in translation [Kraakman *et al*., 1993; Li *et al*., 1999; Ashe *et al*., 2000] until adaptation succeed.
II (≈2 to 60 minutes)	A predominantly transient phase in the regulation of many proteins occurs as a result of rapid signalling events [Gasch *et al*., 2000]. Rehydration begins and cell volume increases (not completed restored) being enhanced by the production and retention of compatible solutes [Albertyn *et al*., 1994a,b; Rep *et al*., 1999]. Ion exclusion systems, namely sodium, are activated. A rapid and reversible change in cytoskeleton organization occurs and proton motive force is restored [Slaninová *et al.*, 2000].
III (≈1 or more hours)	The yeast is fully adapted and registers a state of equilibrated growth. Cell cycle is re-established as a result of a new internal homeostasis. Cell wall and the nucleus are remodelled since DNA/protein synthesis resume according to the expression of salt responsive genes [Hohmann, 2002].

Since most of the osmoregulation studies have been done in the yeast model *S. cerevisiae* [Hohmann and Mager, 1997], the Table refers to events occurring in this yeast as a possible model. As can be observed in Table 1.1 cell suffers many physiological changes after a salt shock, which include mainly: collapse of the osmotic gradient, temporary cessation of growth, loss of an organized actin cytoskeleton, temporary decrease in protein synthesis, induction of a subset of the heat shock proteins, and increases in the concentration of intracellular solutes and macromolecules. In order to grow, cells have to restore their metabolism and thus need to achieve a subtle balance between the need for energy-supplying pathways and expression of genes encoding stress protection/damage repair functions.

Increasing the osmolarity of the medium induces increased expression of many genes as measured by monitoring changes in mRNA or protein levels [Rep *et al*., 2000; Posas *et al*., 2000; Gasch *et al*., 2000; Yale and Bohnert, 2001; Causton *et al*., 2001]. These data revealed that osmotic response is a complex process in which the range of genes expressed depends both on the solute responsible for increasing the osmolarity [Gasch *et al*., 2000], and in the extent of the osmotic shock [Yale and Bohnert, 2001]. More important is the fact that those results suggest that cell is compromised as a whole in the stress response process, and hence, all cellular systems are to some extent implicated.

In fact, alterations in cell volume, cell wall, plasma membrane and cytoskeleton composition in response to salt stress are well documented and will be discussed in detail in Chapter 3. These alterations have serious implications in many cellular functions, such as nutrient transmembrane transport and in the retention of compatible solutes [Beney and Gervais, 2001]. This suggests that not only several cellular systems are implicated but also many different mechanisms are involved in determining the pattern of gene expression under conditions of hyperosmotic stress [Hohmann, 1997, 2002]. These mechanisms relie on signal transduction pathways and their respective regulators components [Hohmann, 1997, 2002].

1.8. Signaling salt stress response

To control gene expression, cell has to sense environmental changes and to transmit a signal to the nucleus [Rep *et al*., 2000]. Yeasts possess rapidly responding, highly complex signaling pathways that allow them to quickly adapt to a changing environment [Ruis and Schüller, 1995; Hohmann, 2000]. Prominent among the yeast signaling pathways are the **M**itogen-**A**ctivated **P**rotein (MAP) kinase cascades that are highly conserved apparently occurring in all eukaryotes [Gustin *et al*., 1998; Posas *et al*., 1998; Nadkarni *et al*., 1999]. MAP pathways relay, amplify and integrate signals from a diverse range of stimuli and elicit an appropriate physiological response through the control of the expression and/or induction of genes. In *S. cerevisiae*, up to the moment, five MAPKs on six functionally distinct pathways are known. These are: the mating pathway, the filamentation-invasion pathway, the morphological switch pathway, the cell integrity pathway (or **P**rotein **K**inase **C** -PKC), the **H**igh-**O**smolarity **G**lycerol pathway (HOG) and the STE vegetative growth pathway [Herskowitz, 1995; Gustin *et al*, 1998; Posas *et al*., 998; Lee and Elion, 1999; Lee *et al*., 1999, Elion, 2000]. At least three signal transduction pathways stimulated by osmotic stress have been characterised in the yeast *S. cerevisiae*: the HOG pathway which responds in a specialized form to an increase in medium osmolarity [Brewster *et al*., 1993; Schüller *et al*, 1994], the Cell Integrity or PKC pathway that responds to a sudden drop in medium osmolarity [Levin and Bartlett-Heubusch, 1992; Paravicini *et al*., 1992; Davenport *et al*., 1995], and the calcineurin mediated pathway activated specifically by salt stress [Maeda *et al*., 1994; Hirayama *et al*., 1995]. A similar

pathway in fission yeast allows adaptation to not only hyperosmotic stress but also other stresses such as heat stress, oxidative stress and nutrient limitation [Kishimoto and Yamashita, 2000]. The stimulation of gene expression after osmotic shock is mediated by different mechanisms [Rep *et al*., 1999; 2000], but the major part of the transcriptional response to osmotic shock is controlled by the HOG pathway and several downstream transcriptional factors [Hohmann, 1997; Gustin *et al*., 1998; Posas *et al*., 1998; Hohmann, 2002]. Other signaling pathways have recently been associated with responses to an osmotic upshift, but there is not yet a consistent knowledge [Tsujimoto *et al*., 2000; Hohmann, 2002].

HOG pathway has actually two branches with different sensitivities to osmotic stress but there is some evidence about the existence of at least one alternative input system [van Wuytswinkel *et al.*, 2000; O'Rourke and Herskowitz, 2002a]. Genetic evidence has placed two proteins, Sln1p (***S****ynthetic* ***l****etal of* ***N****-end rule*) [Posas and Saito, 1997] and Sho1p (***S****ynthetic* ***h****igh* ***o****smolarity sensitive*) [Maeda *et al.*, 1994], upstream of all other HOG pathway components, located in the plasma membrane, and suggested them as probable sensors of these branches. Although observations suggest redundant functions for the two branches, they have been proposed to respond to different degrees of stress. Slnp and Sho1p different sensitivities allow the cell to respond over a wide range of water activity changes [Maeda *et al.*, 1995]. In fact, apparently the cell modulates the response according to stress amplitude only at lower osmolarity and by period of activation at higher osmolarity [Hohmann, 2002]. Thus, the period of Hog1p (a downstream common target of HOG pathway branches) phosphorylation becomes progressively longer as more osmoticum is used to shock the cell, *e.g*., 1.4 M NaCl [Rep *et al*., 1999]. On the other hand, at lower osmolarity (< 0.3 M NaCl) the cell will activate one or other branch to respond more effectively, but the Hog1p phosphorylation will not change [Gasch *et al*., 2000]. However, it is also apparent that the HOG pathway is active even without osmotic stress, which could be a reflection of its role in monitoring turgor during cell growth [Cullen *et al*., 2000; Lee *et al*., 1999a,b]. Together, both branches of the HOG pathway orchestrate osmotic responses and integrate the need for cell expansion in response to osmotic signals generated by growth and by the environment [Hohmann, 2002]. The HOG pathway is not only required for various responses to hyperosmotic stress, but also hyperosmotic stress activates the pathway. This pathway is activated by NaCl, KCl, sorbitol or glucose [Brewster *et al.*, 1993; Davenport *et al.*, 1995], showing that the activating stimulus is related to the osmotic change rather than an increase in concentration of a specific solute [Tamás *et al*., 2000]. Recently Winkler and collaborators (2002) found that HOG pathway is also activated by heat stress in a Sho1p dependent manner.

During salt stress, homeostasis mechanisms namely those involving ions extrusion are fulcral in cell detoxification. Calcineurin dependent signaling pathway has also been implicated in salt stress response. This pathway is activated, in *S. cerevisiae*, by a series of events, namely an increase in the level of extracellular ions such as Na^+, Li^+ and Ca^{2+} [Nakamura *et al*., 1993; Mendoza *et al*., 1994; Aramburu *et al*., 2000; Rusnak and Mertz, 2000]. The role of calcineurin in ion homeostasis is effected by controlling *PMC1* and *PMR1*, which encode P-type ATPases that mediate transport of calcium ions between different cellular compartments [Cunningham and Fink, 1996], *ENA1 (PMR2)* a P-type Na^+-ATPase [Haro *et al.,* 1991; Márquez and Serrano, 1996; Mendoza *et al.,* 1994], and *TRK1* and *TRK2*, which encodes K^+ uptake systems [Serrano *et al*., 1999]. Sodium tolerance in yeast is enhanced by continuous activation of calcineurin, which is required for modulation of the Na^+ efflux mechanisms [Park *et al*., 2001]. Defects in calcineurin signaling pathway do not cause an apparent phenotype directly related to osmotic stress, but this pathway contributes to recovery from NaCl-induced osmotic stress and interacts with pathways that mediate cell surface remodeling (like cell wall metabolism) in response to external

signals, such as turgor increase and mating pheromone [Hohmann, 2002].

Cell sensing and mechanisms of signal transduction trigger specific and general stress responses that are dificult to separate, since often they respond to different type of stress responses. This phenomenon probably reflects the need for cells to repair specific primary damage and to adjust simultaneously various metabolic disturbances when exposed to a particular stressing agent. Thus, an individual stress agent like salt can cause cellular damage either directly or indirectly through various modes, *e.g.*, direct damage to membrane or inhibition of enzyme activity, plus secondary oxidative damage through formation or accumulation of reactive oxygen species, and this might require a diversified response by the cell. Yeasts have a global mechanism for stress tolerance acquisition that protects cells from a number of different stress conditions, which has been termed General Stress Response (GSR) [Hohmann, 1997], common environmental response [Causton *et al*., 2001] or environmental stress response (ESR) [Gasch *et al.*, 2000]. GSR is characterized by genes whose expression is induced by hyperosmotic stress but also by other stress conditions such as heat stress, oxidative stress, nutrient starvation, weak acids and probably any condition that cause slow-down or arrest of cell proliferation [Hohmann, 1997]. Global gene expression has shown that expression of more than 10% of yeast genes is affected by several unrelated stress conditions, such as nutrient starvation, oxidative stress, heat shock and hyperosmotic stress [Causton *et al*., 2001]. Since GSR occurs simultaneously with specific responses to a sudden stype of stress [Rep *et al*., 2000], some authors proposed that GSR is no more than a general adaptative response to suboptimal environments [Gasch *et al*., 2000]. In fact, upon challenge by increases in molarity, the expression of general responsive genes such *HSP12*, encoding a heat shock protein, and *CTT1*, encoding the Catalase T enzyme, are induced, suggesting that cytoskeleton alterations and oxidative species occur under osmotic stress [Varela *et al.*, 1992; Hirayama *et al.*, 1995; Rep *et al*., 2000; Causton *et al*., 2001; Yale and Bohnert, 2001].

Cells submitted to osmotic up-shifts, before adapting to a new steady-state, will respond with alterations in gene expression that may be transient or durable [Yale and Bohnert, 2001]. Larger shifts in environmental changes result in prolongated and larger alterations, *i.e.*, the duration and amplitude of the transient changes in transcript levels vary with the magnitude of the environmental changes [Gasch *et al*., 2000; Hohmann, 2002].

1.9. Transcriptional and proteomic response of *S. cerevisiae* to salt stress

Yeasts, namely *S. cerevisiae*, have been considered an excellent model for the study of the mechanisms underlying tolerance to saline stress, particularly because it has been shown that fungi and higher plants not only have similar ion transport systems at their plasma membranes [Serrano *et al*., 1997], but they also share similar cation detoxification mechanisms [Gaxiola *et al*., 1999], and, most probably, signal transduction pathways [Lee *et al*., 1999b; Pardo *et al*., 1998]. Thus the physiological and molecular response of *S. cerevisiae* to hyperosmotic stress has for long been a subject of study and, at present, there is substantial data on the transcriptional expression of several genes and the expression of important proteins associated with growth in the presence of salt [Blomberg and Adler, 1992; Norbeck and Blomberg, 1996, 1997a,b; Hohmann, 1997; Posas *et al.*, 2000; Gasch *et al*., 2000; Rep *et al*., 2000; Yale and Bohnert, 2001; Hohmann, 2002].

Genetic expression can be regulated at several levels, such as during and after the transcription of genes and their translation to proteins [Madigan *et al*., 1997]. On the other hand, protein

function and localization as well as enzyme activity may also be regulated in many ways by mechanisms that activate, repress, derepress or lead to their degradation [Madigan *et al*., 1997]. Due to these aspects, results concerning the transcription of genes should be corroborated by measurement of protein expression levels and protein activity whenever possible [Hohmann, 2002].

It was estimated that *S. cerevisiae* contained roughly 200 proteins, which respond to salt stress [Blomberg, 1995], but only 18 to 30 proteins increased considerably their expression when cells were shocked for 1 hour with 0.7 and 1.4 M NaCl, respectively [Blomberg, 1995; Norbeck and Blomberg, 1996, 1997b] (Table 1.2).

Table 1.2. Relevant genes whose proteins have been reported as bein up- or down-regulated by hyperosmotic stress [Norbeck and Blomberg, 1996; 1997b]

Gene	Gene product
Up-regulated	
CTT1	Catalase T
DAK1	Dihydroxyacetone kinase
ENO1	Enolase I
GCY1	Putative glycerol dehydrogenase
GPD1	Glycerol-3-phosphate dehydrogenase
GPP1/2	Glycerol-3-phosphatase I and II
HSP104	Heat shock protein
TDH1	Glyceraldehyde-3-phosphate dehydrogenase I
Down-regulated	
ADH1	Alcohol dehydrogenase I
ALD6	Aldehyde dehydrogenase
ENO2	Enolase II
GDH1	Glutamate dehydrogenase (NADP)
HXK2	Hexokinase pII
MET6	Methionine synthase
PDC1	Pyruvate decarboxylase
TDH3	Glyceraldehyde-3-phosphate dehydrogenase III

Upon osmotic shock yeast cells synthesize and accumulate glycerol, a so-called compatible solute or osmolyte that may stabilize soluble enzymes and restore the cell turgor pressure required for growth [Brown, 1978]. It is likely, therefore, that some of the proteins showing increased synthesis rates upon osmotic shock are enzymes involved in glycerol metabolism. In fact, this was suggested years ago on the basis of the experiments of Trollmo and collaborators (1988), Blomberg and Adler (1989) and André and collaborators (1991), who not only showed that some enzyme activities for glycerol production were induced upon a shift to lower water activity, but also pointed to the fact that full induction of tolerance to high salt was dependent on *de novo* protein synthesis. In fact, Gpd1p, the key enzyme in glycerol production is among the highly NaCl-responsive proteins identified in Table 1.2 besides Hsp104p, and Ctt1p. Furthermore, Gdp1p, Gpp2p, Gcy1p and Dakp, related with glycerol production and/or consumption, some glycolytic enzymes like Fba1p (Fructose biphosphatase), Tdh3/2p, Eno1/2p, Pykp (pyruvate kinase) and ethanol producing enzymes such Pdc1p and Adh1p were also identified but were down-regulated [Norbeck and Blomberg, 1996, 1997b] (Table 1.2). It was the first time that proteins with homology with the glycerol dehydrogenase and

dihydroxyacetone kinase found in other yeasts like *D. hansenii* [Adler *et al*., 1985] and *Z. rouxii* [van Zyl *et al*., 1991], where identified in *S. cerevisiae*. Although the rate of protein synthesis increased for both of these proteins, it was only possible to determine specific activity for the dihydroxyacetone kinase [Norbeck and Blomberg, 1997]. An importante feature encountered by Norbeck and Blomberg (1997b) was the selective expression of isogenes. Some genes encoding isoenzymes such as *GLK1/HXK2*, *ENO1/ENO2*, *GPD1/GPD2*, *DAK1/DAK2*, *GCY1/YPR1* and *ALD5/ALD6*, displayed different regulation by salt stress of each gene of the pair. The general pattern is for one isogene to be regulated while the other shows roughly constant expression. Besides the level of protein synthesis, Norbeck and Blomberg (1997b) also evaluated the *GPD1*, *GPP1/2*, *ENO1/2* and *DAK1* respective mRNA levels by Northen analysis. In fact, mRNA levels of the genes tested confirmed data from Table 1.2, suggesting that these genes regulation is mainly effected at the transcription level. Moreover, for dihydroxyacetone kinase they even determined specific activity, which again confirmed the increased value observed for protein and mRNA in salt shocked cells. At salt concentrations of 1.4 M NaCl, induction was almost exclusively the mode of regulation encountered [Norbeck and Blomberg, 1997b]. Furthermore, the repressed proteins still have a good background level, and thus this could implicate that under stress there is mainly a need for induction and the addition of new metabolic pathways rather then the repression and the silencing of existing ones [Norbeck and Blomberg, 1997b].

Gene transcription response to stress has been lately intensively explored and, in spite of the number of genes expressed after an osmotic shock being different in different publications, the type o genes expressed is quite similar [Rep *et al*., 2000; Posas *et al*., 2000; Gasch *et al*., 2000; Yale and Bohnert, 2001; Causton *et al*., 2001] (Table 1.3). As can be observed from Table 1.3, cells respond to saline stress by inducing and repressing the expression of a very large number of genes and this suggests, as said before for stress signaling, that stress adaptation requires and involves the regulation of many cellular aspects. Genes whose expression is adjusted under stress belong largely to three classes (I) genes whose product is involved in protein production and their expression, (II) genes involved in cell protection from oxidative damage and protein denaturation, and (III) genes encoding enzymes responsible for redox, energy and carbohydrate metabolism [Gash *et al*., 2000; Rep *et al*., 2000; Causton *et al*., 2001]. Though this might seem an oversimplified interpretation, metabolic adjustments tend to the optimization of the use of the available resources and accumulation of reserves and stress protectants [Hohmann, 2002]. Indeed, mRNA expression of genes encoding proteins required for gene expression, protein production, amino acid metabolism, cell wall maintainence, nucleossome structure, DNA synthesis and nucleotide metabolism was observed to be diminished upon stress, although sometimes only transiently [Rep *et al*., 2000; Causton *et al*., 2001]. For instance, repression of the ribosomal protein genes upon stress treatment as been known for many years, and was confirmed recently in mRNA expression studies [Herruer *et al*., 1988; Yale and Bohnert, 2001]. Nevertheless it should be safeguarded the transient nature of this regulation, in the sense that mRNA production can increase for a small period of time and decrease for longer periods as stated by Yale and Bohnert (2001).

Genes whose expression drops rapidly under salt stress, have been shown to belong to amino acid metabolism, cell wall maintenance, nucleosome structure, DNA synthesis and nucleotide metabolism [Gasch *et al*., 2000; Causton *et al*., 2001]. These processes are in less demand when cells do not proliferate, and hence the drop in expression may reflect a temporary proliferation arrest upon a sudden stress or a slowdown of the proliferation rate under mild stress [Hohmann, 2002]. While an overall reduction of protein synthesis may be well compatible with a transient inhibition of cell growth and proliferation, the expression of genes encoding important functions for stress adaptation is stimulated,

and their translation must be ensured [Hohmann, 2002].

In general, transcriptional response of *S. cerevisiae* to saline stress induces the expression of genes responsible for glycerol production, being *GPD1* one of the most studied (Table 1.3). The results concerning Gpd1p expression presented in Table 1.2 were also confirmed at transcription expression level [Varela *et al.*, 1992; Albertyn *et al.*, 1994b; Hirayama *et al.*, 1995].

Table 1.3. Relevant genes of *S. cerevisiae* identified in literature whose mRNA level is up- or down regulated after a shift to high osmolarity.

Gene	References
Up-regulated	
GPD1 Glycerol-3-phosphate dehydrogenase	Varela *et al.*, 1992; Albertyn *et al.*, 1994; Hirayama *et al.*, 1995; Norbeck and Blomberg 1997; Rep *et al*., 2000; Posas *et al*., 2000; Yale and Bohnert, 2001
HSP26/12 -Heat-shock proteins	Varela *et al* ., 1992; Hirayama, *et al.*, 1995; Causton *et al*., 2001; Rep *et al.*, 2000;Yale and Bohnert, 2001
HSP42/78/104 -Heat-shock proteins	Causton *et al*., 2001; Rep *et al*., 2000; Posas *et al*., 2000; Yale and Bohnert, 2001
ENA1-P-type (Na^+,Li^+,K^+) ATPase	Garciadeblás *et al*., 1993; Hirayama *et al.*, 1995; Rep *et al*., 2000; Posas *et al.*, 2000;
GLK1 -Glucokinase	Hirayama *et al.*, 1995; Rep *et al*., 2000; Posas *et al*., 2000; Yale and Bohnert, 2001
HXT1 -Hexose transporter	Hirayama *et al.*, 1995; Rep *et al*., 2000; Posas *et al*., 2000; Yale and Bohnert, 2001
GPP1/2 -Glycerol-3-phosphatase I and II	Norbeck and Blomberg 1997b; Rep *et al*., 2000
ENO1 -Enolase I	Norbeck and Blomberg 1997b
DAK1 -Dihydroxyacetone kinase	Norbeck and Blomberg 1997b; Rep *et al*., 2000
TPS1-3,TSL1 -Subunits of trehalose synthase	Causton *et al*., 2001; Rep *et al*., 2000; Posas *et al*., 2000; Yale and Bohnert, 2001
PHB2, RPN5,UBC5/8, YPS6 Gene products involved in protein degradation	Causton *et al*., 2001
CTT1 -Catalase T	Causton *et al*., 2001; Rep *et al*., 2000
TTR1 –Glutaredoxin; *TRX2* -Thioredoxin	Causton *et al*., 2001; Rep *et al*., 2000; Yale and Bohnert, 2001
GRE2 -Unknown function	Rep *et al*., 2000; Posas *et al*., 2000
GLO1 Glyoxylase	Rep *et al*., 2000
PUT4 –Proline permease	Rep *et al*., 2000; Posas *et al*., 2000; Yale and Bohnert, 2001
ALD6	Akhtar *et al*., 1997; Posas *et al*., 2000
ALD2	Miralles and Serrano, 1995; Posas *et al*., 2000
VMA5/6/7, VPH1, TFP1 Components of the vacuolar H^+-ATPase	Posas *et al*., 2000
TIF1/2, PRT1, TIF11, HYP2, CDC95 Subset of translation intiation factors	Posas *et al*., 2000
Down-regulated	
Cytoplasmic ribosomal proteins	Causton *et al*., 2001
Polymerase I,II and III transcrition	Causton *et al*., 2001
tRNA synthetases	Causton *et al*., 2001
Proteins required for processing rRNAs	Causton *et al*., 2001
TIF35 Subset of translation intiation factors	Causton *et al*., 2001; Rep *et al*., 2000
ADH2 Alcohol dehydrogenase	Rep *et al*., 2000
PMA Plasma membrane proton ATPase	Rep *et al*., 2000
PMP2–Plasma membrane associated with *PMA1*	Rep *et al*., 2000
GND1 -6-phosphogluconate dehydrogenase	Rep *et al*., 2000
PHO88 -Regulator of phosphate transporter	Rep *et al*., 2000

Transcriptional expression of genes encoding enzymes involved in trehalose metabolism, *GLK1*, *PGM2*, *HXK1*, *TPS1*, *TPS2* and *NTH1* were also highly increased after a salt shock (Table 1.3 and Figure 1.1). Nevertheless trehalose does not accumulate to osmotically significant concentrations like glycerol does according to its implication in yeast stress response as osmoprotectant [Parrou *et al*., 1997; Hounsa *et al*., 1998]. Since the enzymes involved in treahalose breakdown and formation of glucose (trehalases encoded by *NTH1* and *ATH1*) were also increased upon salt stress, they may complete a cycle in which substantial amounts of trehalose are not allowed to accumulate under saline conditions [Parrou *et al*., 1997; Zähringer *et al*., 1997]. Genes with transport functions were only induced in longer periods of exposition to stress being the glucose transporter encoded by *HXT1* and the aminoacids transporters of proline, uracil and leucine some examples. Among the ion transporters only those related with Na^+ extrusion seems to increase their transcript levels [Garciadeblás *et al*., 1993; Hirayama *et al*., 1995; Rep *et al*., 2000; Posas *et al*., 2000]. In fact, H^+-ATPase *PMA1* was not induced, but the vacuolar H^+-ATPase did, pointing to the importance of the vacuole in salt stress resistance presented before by Latterich and Watson (1993).

Both global gene [Rep *et al*., 2000; Posas *et al*., 2000; Gasch *et al*., 2000; Yale and Bohnert, 2001; Causton *et al*., 2001] and protein [Blomberg 1995, 2000; Norbeck and Blomberg, 1996, 1997b] expression studies have confirmed and extended earlier findings that the enzyme composition in the glycolytic pathway as well as the pathways connected to it, changes under hyperosmotic stress conditions. Fairly little is known about how much these expression changes truly affect flux through metabolic pathways [Hohmann, 2002]. However, because glycolysis is the central metabolic pathway in *S. cerevisiae*, stress-induced adjustments of glycolysis appear to be central to controlling the adaptation of metabolism to stress. Transcriptional response of the genes encoding enzymes from important metabolic pathways was temptatively organized in Figure 1.1 [Norbeck and Blomberg, 1996, 1997b; Rep *et al*., 2000; Posas *et al*., 2000; Gasch *et al*., 2000; Yale and Bohnert, 2001; Causton *et al*., 2001]. As can be seen most of the genes are induced upon a salt shock (the expression intensity and stress level was not discriminated). The Figure 1.1 intends to show as a whole and in a simplified way, the expression genes implicated in metabolic functions common in yeast, like glycolysis, pentose pathway, trichloroacetic acid cycle, glycogen, glycerol, acetic acid, ethanol and trehalose synthesis. Though transcriptional gene expression is not fully complemented with the same results in protein expression and in enzyme activity, it suggests that metabolism is indeed induced upon a hyperosmotic shock (Figure 1.1).

Recently the transcriptional response of *S. pombe* was studied and revealed the increase in genes whose expression is also increased in *S. cerevisiae,* such as neutral trehalase (*NTP1*), trehalose-phosphate synthase (*TPS1*), glucose-6-phosphate dehydrogenase (*ZWF1*), *GPD1*, *GUT1*, alcohol dehydrogenase, phosphoglycerate mutase and malate dehydrogenase [Chen *et al*., 2003]. These results suggest that some cellular aspects might be common among the salt stress response in yeasts.

Some general conclusions can be withdrawn from the data recently published on stress response to hyperosmotic stress in *S. cerevisiae*: (i) The set of genes whose expression responds specifically to hyperosmotic shock but not to any other type of stresses is rather small, and within this group most expression changes are not very strong and many genes are still uncharacterized; (ii) Both stimulation of expression and diminished expression of different genes seem to be important aspects of the response of *S. cerevisiae* to stress, being the number of genes whose expression drops apparently higher than that of those whose expression increases [Norbeck and Blomberg, 1997b; Rep *et al*., 2000; Posas *et al*., 2000; Yale and Bohnert, 2001; Hohmann, 2002].

Although several studies on transcriptional and protein expression have been recently

published they are of limited conclusion in what concerns hyperosmotic salt stress response [Norbeck and Blomberg, 1996, 1997; Rep *et al*., 2000; Posas *et al*., 2000; Gasch *et al*., 2000; Yale and Bohnert, 2001; Causton *et al*., 2001]. These studies focused on the early phase of the response to osmotic shock, and none of them explored the expression pattern of cells actively growing in high-osmolarity medium or pre-adapted to grow at lower osmoticum concentrations. Further studies on longer periods of time with higher levels of stress and using paralely shocked and adapted cells are missing and are of high importance for better understanding of salt stress response and this needs to be carefullu indexed to the question of the strain, media and growth conditions.

Figure 1.1. Scheme of the regulation of transcripts in the glycolytic pathway, trichloroaceticacid cycle, pentose phosphate pathway and pathways for production of glycerol, mannitol, trehalose, glycogen, ethanol and acetate in *S. cerevisiae*. Expression of genes encoding the proteins that are highlighted by a black open box is stimulated after osmotic shock, and expression of those inside an open box is diminished [Norbeck and Blomberg 1997b; Rep *et al*., 2000; Posas *et al*., 2000; Yale and Bohnert, 2001; Hohmann, 2002].

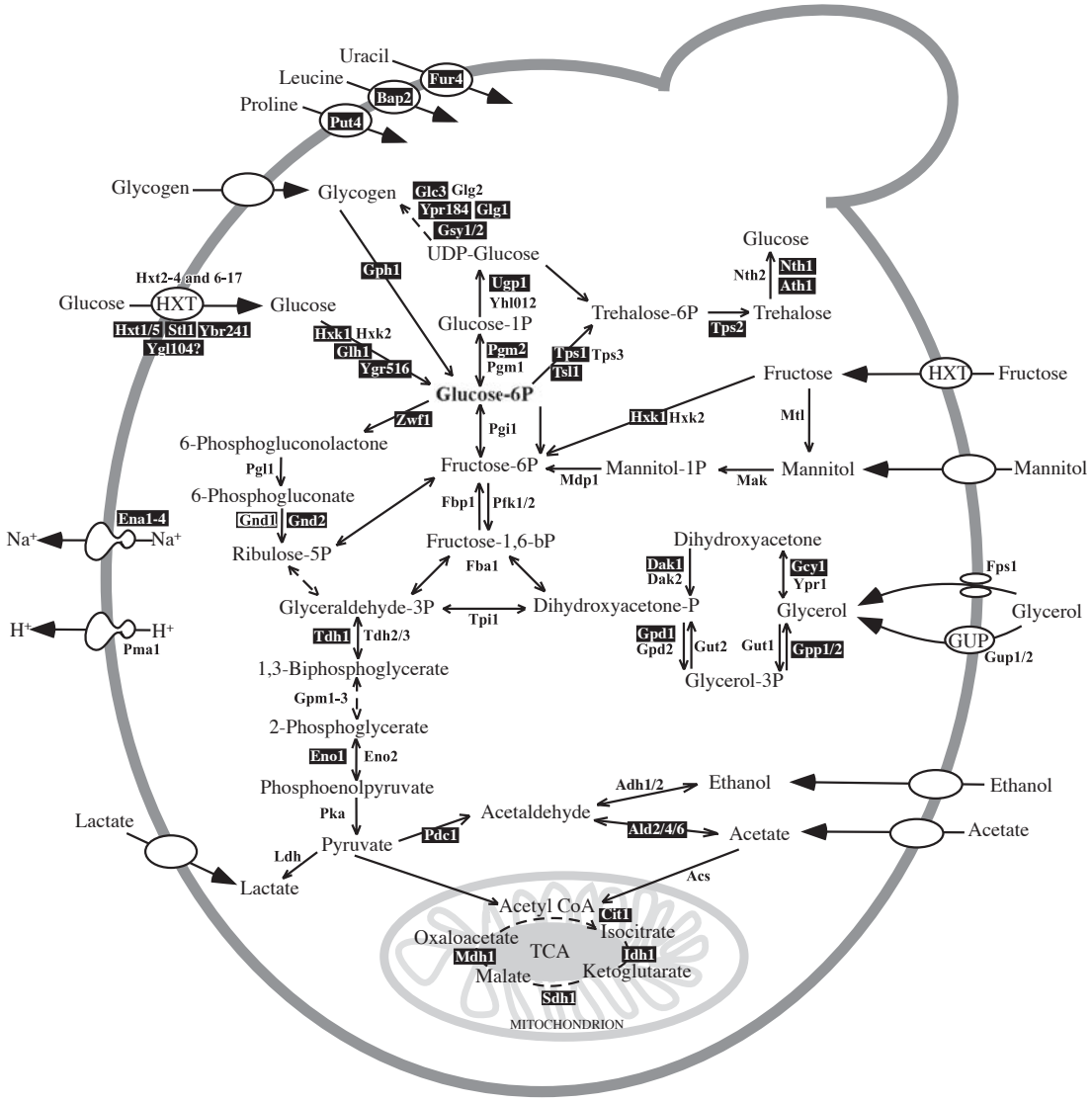


Table 1.4. Listing of the genes and their respective product represented in Figure 1.1.

Gene name	Description of gene product	Major function
ACS	Acetil CoA synthetase	
ADH1	Alcohol dehydrogenase isoform 1	
ADH2	Alcohol dehydrogenase isoform 2	Gluconeogenesis; glucose repressible
ALD2, ALD4	Aldehyde dehydrogenase (NAD(P)) mitochondrial	
ALD3, ALD6	Aldehyde dehydrogenase (NAD(P)) cytosolic	
ATH1	Trehalase	Trehalose metabolism
BAP2	Leucine permease	
CIT1	Citrate synthase mitochondrial	Tricarboxylic acid cycle
DAK1, DAK2	Dihydroxyacetone kinase	Glycerol breakdown
ENA1	P-type ATPase involved in sodium and lithium efflux	Required for sodium tolerance
ENO1, ENO2	Enolase isoforms 1 and 2	Lower part of glycolysis
FBA1	Fructose-1,6-bisphosphate aldolase	Glycolysis
FBP1	Frutose-2,6-biphosphatase	Glycolysis
FPS1	Glycerol channel	Glycerol efflux upom osmotic shock
FUR4	Uracil permease	
GCY1, YPR1	Glycerol dehydrogenase	Glycerol utilization
GLC3	Alpha-1,4-glucan branching enzyme	Glycogen production
GLK1	Glucokinase	Glycolysis
GND1, GND2	6-Phosphogluconate dehydrogenase (NADP)	Pentose phosphate pathway
GPD1, GPD2	Glycerol-3-phosphate dehydrogenase isoform 1 and 2	Glycerol production
GPH1	Glycogen phosphorylase	Glycogen metabolism
GPM	Phosphoglycerate mutase	Lower part of glycolysis
GPP1,GPP2	Glycerol-3-phosphatase isoform 1 and 2	Glycerol production
GSY1,GSY2	Glycogen synthetase isoform 1 and 2	Glycogen production
GUP1,GUP2	Glycerol uptake isoforms 1 and 2	Involved in glycerol active uptake
GUT1	Glycerol kinase	Glycerol utilization
GUT2	Glycerol-3-phosphate dehydrogenase mitochondrial	Glycerol utilization
HXK1, HXK2	Hexokinase isoforms 1 and 2	Glycolysis
HXT1-17	Member of the sugar permease family	Sugar uptake
STL1	Member of the sugar permease family	Sugar uptake
IDH1	Isocitrate dehydrogenase	Tricarboxylic acid cycle
LDH	Lactate dehydrogenase	
MAK	Mannitol kinase (putative)	Mannitol utilization
MDH1	Malate dehydrogenase	Tricarboxylic acid cycle
MPD1	Mannitol-1-phosphate dehydrogenase	Mannitol production
MTL	Mannitol dehydrogenase (NAD)	Mannitol utilization
NTH1	Neutral trehalase	Trehalose breakdown
PDC?	Pyruvate dehydrogenase complex	
PDC1	Pyruvate descarboxylase	Lower part of glycolysis
PFK1, PFK2	Phosphofructokinase isoforms 1 and 2	Glycolysis
PGI1	Glucose-6-phosphate isomerase	Glycolysis
PGL1	6-Posphogluconolactonase	Pentose phosphate pathway
PGM2	Phosphoglucomutase major isoform	Trehalose and glycogen production
PKA	Pyruvate kinase	Lower part of glycolysis
PMA1	Plasma membrane proton ATPase	
PUT4	Proline permease	Amino acid utilization
SDH1	Flavoprotein subunit of succinate dehydrogenase	Tricarboxylic acid cycle
TDH1-3	Glyceraldehyde-3-phosphate dehydrogenase	
TPI1	Triose phosphate isomerase	Glycolysis
TPS1	Trehalose-6-phosphate synthase	Trehalose production
TPS2	Trehalose-6-phosphatase	Trehalose production
TSL1	Subunit of trehalose synthase complex	Trehalose production
UGP1	UDP-glucose pyrophosphorylase	Trehalose and glycogen production
YGL104C	Member of the sugar permease family	Sugar uptake
YBR241	Member of the sugar permease family	Sugar uptake
ZWF1	Glucose-6-phosphate dehydrogenase	Glycolysis

1.10. Salt stress resistance levels

Microbial life can be found over the whole range of salt concentrations from freshwater and marine biotopes to hypersaline environments with NaCl concentrations up to saturation [Brown, 1976, 1978; Tokuoka, 1993; Buchalo *et al*., 1998, Ventosa *et al*., 1998; Horikoshi and Grant, 1998; Almeida, 1999; Oren, 1999, 2002; Antón *et al*., 2000; Gunde-Cimerman *et al*., 2000; Sleator and Hill, 2001; Lahav *et al*., 2002; Roberts, 2004]. Halophilic and halotolerant microorganisms are found in all three domains of life: *Archaea*, *Bacteria*, and *Eukarya* [Buchalo *et al*., 1998; Antón *et al*., 2000; Oren, 1999, 2002]. As with all extreme environments, microbial diversity decreases as the water stress of an aqueous environment increases (a_w decrease) and only a few yeast species grow under conditions of extreme water stress [da Costa and Nobre, 1989; Almeida, 1999]. However, colonization of hypersaline environments such as salt lakes [Buchalo *et al*., 1998; Gunde-Cimermann, *et al*, 2000] and salted food products [Onishi, 1957; Andrews and Pitt, 1987; Hocking, 1988; Takashina *et al*, 1994] is often highly successful, and salt-loving and/or salt-tolerant microorganisms may reach high population densities in such ecosystems [Oren, 1999].

The ability to withstand stressful environments is largely correlated with the mechanisms of sensing and responding of a cell and their efficiency can determine the robustness and tolerance levels of a yeast strain. Several attempts have been made to classify species according to their ability to grow below some defined threshold of a_w. Growth studies have been used frequently with the purpose to determine experimentally the degree of tolerance [Onishi, 1957; van Eck *et al*., 1993; Tokuoka and Ishitani, 1991; Lages *et al*., 1999]. As a consequence, different terms have been used in the literature to describe the overall response of organisms to low availability of water, such as "osmotolerant", "xerotolerant", "xerophilic", "osmophilic", "xerophilic", "salt-loving", "halophilic", "halotolerant" [Brown, 1976, 1978; Hocking, 1988]. Some researchers have restricted, and thus oversimplified, the terminology to two main classes: tolerant and non-tolerant. These can be used as a relatively qualitative indicator, yet, with the raising of knowledge on microorganisms exhibiting different tolerance levels, a more precise classification is needed. The term osmophilic was given by van Richter (1912) to the group of yeasts that can grow well in environments with high pressures, and thus those showing sugar- and salt-tolerant properties have been traditionally called osmophilic or osmotolerant. These terms are not always considered to be accurate, essentially because it has been shown that the sugar and salt-tolerance of some yeast like for example *Z. rouxii* differ considerably, and also because there is a high variation from strain by strain, according to their origin [Onishi, 1957; van Eck *et al*., 1993]. Furthermore, most of the so-called osmophilic yeasts do not require low a_w to grow [Hocking, 1988, Tokuoka, 1993; Lages *et al*., 1999]. Therefore it was recommended that, instead they should be designated as sugar-tolerant or salt-tolerant [Tokuoka, 1993]. The term halophilic or halophile (salt-lover) has been generally restricted along time to those microorganisms that actually require NaCl for growth, being until present assigned only to members of the bacteria and *Archae* group [Brown, 1978]. Of the other terms cited above, xerophilic (from the Greek dry –loving) seems to be the most appropriate, as it describes the preference or requirement for reduced water activity and not for increased osmotic pressure as is implied by the term osmophilic [Hocking, 1988].

There are many groups of microorganisms that are able to grow in the presence of salt, but only the yeasts and filamentous fungi have so far mastered the art of growth in environments containing high sugar concentrations [Hocking, 1988, Tokuoka, 1993]. Thus, those organisms that are able to grow under higher osmotic pressure like those created by highly concentrated sugar solutions, are termed osmotolerant. For example, *Z. rouxii* is a osmotolerant yeast well known for its ability to grow in food with low a_w, being frequently termed a food spoilage microorganism [Thomas and

Davenport, 1985; Pitt and Hocking, 1997]. The intrinsic a_w of food products is a very important aspect of food preservation. The growth of various microorganisms stops at a given level of water activity and a comprehensive knowledge of these is essential for food industry as well as for research purposes as said before in section 1.3. Osmotolerant microorganisms are able to grow in media with a_w as low as 0.65 [van Eck *et al*., 1993], and as said above, are restricted to the fungi and yeast groups.

In Table 1.5 and Figure 1.2 are presented the terms published by Kushner (1978, 1993), which aimed to establish classes of salt tolerance considering the salt requirement of microorganisms. According to Figure 1.2 only those microorganisms requiring a certain amount of salt to grow and having an optimum growth rate in the presence o salt are included in halophilic categories. Differences between halophiles and extremely halophiles lay on the amount of salt necessary to grow. Generaly, extremely halophilic organisms require a minimum of 2.5M of NaCl to grow, while halophiles require lower amounts to growth (Table 1.5).

Table 1.5. Classification of microorganisms according to salt resistance [Kushner 1978, 1993].

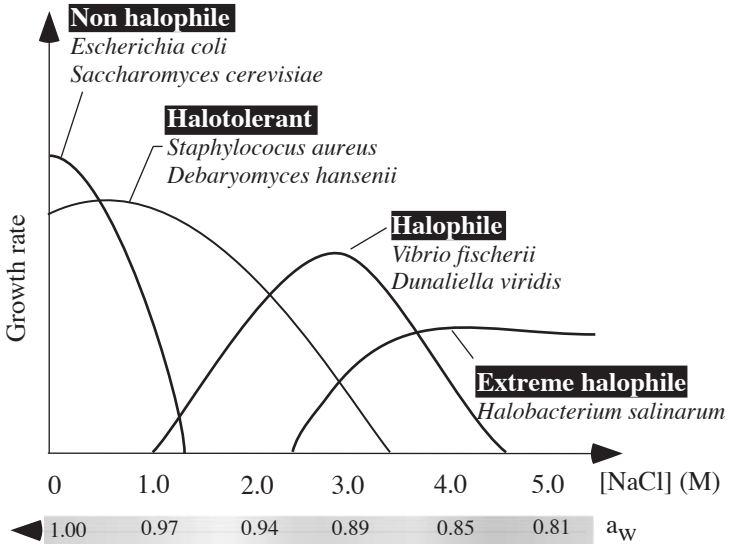
	Salt concentration (M)	
Category	Range	Optimum
Nonhalophile	0-0.1	<0.2
Slight halophile	0.2-2.0	0.2-0.5
Moderate halophile	0.4-3.5	0.5-2.0
Borderline extreme halophile	1.4-4.0	2.0-3.0
Extreme halophile	2.0-5.2	>3.0
Halotolerant	0->1.0	<0.2
Haloversatile	0->3.0	0.2-0.5

Following the reasoning above, although not suggested by the author, extremely halotolerant organisms should also exist just differing from the halotolerant in the salt concentration that allows the maximal growth rate. This way, extremely halotolerant organisms will be those whose optimum salt concentration for growth can reach 2.5M NaCl or more, and halotolerant those whose optimum growth rate is established below 1M NaCl. In fact, the unusual group capable of growing over a range of salt concentrations from zero to saturation, with optimum growth rate in the presence of salt, have been given the name of haloversatile [Horikoshi and Grant, 1998], or euryhaline [Vreeland, 1987] by other authors. Nevertheless, salt tolerance can vary widely depending on the environmental conditions such as pH, temperature, nutrients availability and other. Inevitably, there are microorganisms that do not fit exclusively into any of the category proposed in Figure 1.2. Thus, Figure 1.2 is intended to give just a series of more and less concessus terms on tolerance levels classification of microorganisms with respect to salt.

Nowadays it is still difficult to establish classes of resistance with well defined limits in a_w terms like those represented in Figure 1.2, as more knowledge is emerging more we are faced with new microorganims and also new strains closely related taxonomically behaving phisologically very differently. Indeed, the extreme halophily attributed only to the prokaryotic group of halobacteria (extremely halophilic *Archae*) is actually being shared by black yeasts, *e.g.*, *H. werneckii* [Gunde-

Cimerman, *et al*., 2000] and some filamentous fungi [Andrews and Pitt, 1987; Buchalo *et al*., 1998], that have been isolated from salt fish and from hypersaline waters in salterns. The reasons why authors have classified some black yeasts and some fungi as truly halophilic is quite different, for example, from the ones utilized by other authors to classify (or propose) the halotolerant yeast *D. hansenii* as halophilic [Prista *et al*., 1997]. Black yeasts were said to be halophilic because they are able to live in the presence of NaCl up to 30%, being found in the salterns during the period of maximal rate of salt formation [Gunde-Cimerman, *et al*., 2000], *i.e.*, saturation.

Figure 1.2. Scheme representing growth rate patterns of salt resistance categories adapted from Horikoshi and Grant, (1998) and Madigan and collaborators (1997).



In the case of the filamentous fungi isolated from salt fish [Andrews and Pitt, 1987] and from the extremely hypersaline water of the Dead Sea [Buchalo *et al*., 1998], they were said to be halophilic because they do not grow in the absence of some salt and have an optimum growth rate in the presence of 0.5 to 2M NaCl. On the other hand, *D. hansenii* was suggested to be halophilic because: (i) it has a maximum growth rate in the presence of 0.25-0.5M NaCl [Prista *et al*., 1997], (ii) its metabolism is less sensitive to salt allowing higher internal sodium concentrations than other yeasts [Prista *et al*., 1997], and (iii) it has been shown to have an ability to switch between sodium and potassium ions according to their availability in the medium, not being subjected to Na^+-toxicity as other yeasts [Prista *et al*., 1997]. *Candida halonitratophila* and *Candida versatilis* were also classified as halophilic yeasts by Barnett and collaborators (1990), because within their experimental frame, these yeasts were only able to grow in media with some salt, nevertheless they have been shown to grow in the absence of salt by Lages and collaborators (1999). Intuitively the term halophile refers to those organisms that require a certain amount of salt to grow, but in fact it seems that the term has not been

always applied within that definition. This way, with no clear systematic criteria available, it is important to set out the implications of the term halophilic or halophile when it is being used to classify a microorganism.

Yeasts form a heterogenous group that may colonize several environments within a large range of salt concentrations from salty foods and their ingredients [Tokuoka and Ishitani, 1991; Pitt and Hocking, 1997; Boekout and Robert, 2003] to salterns and lakes [Almeida, 1999; Gunde-Cimermann *et al*., 2000]. The diversity in the properties of these habitats is reflected in the great diversity among the yeasts resistance capabilities, *i.e.*, their physiology, even between those highly related phylogenically such as strains of the same specie [Blomberg, 1997]. Indeed, different studies [Tokuoka and Ishitani, 1991; van Eck *et al*., 1993] on several strains isolated from different environments proved that the intrinsic type of resistance found for each strain is in direct relation with the type of media from which they were isolated. They found yeasts strains belonging to the same species such as *Z. rouxii* that when isolated from soy sauce, miso or molasses (food with high salt content) resist both to high salt and to high sugar contents; but when isolated from food whith high sugar contents are only able to growth in media with high sugars content (sugar-tolerants). These observations agree with the idea reported many years ago by Onishi (1957) that the tolerance differs considerably strain by strain according to their origin. [Tokuoka and Ishitani, 1991]. This versatility of yeasts makes them serious food spoilage microorganisms [Boekout and Robert, 2003]. Bacteria are not usually related with resistance to sugars, although they are able not only to spoil, but even to poison food products.

1.11. Scope and main goals of this thesis

Yeasts are widespread in the natural environment, and can be isolated from different habitats like plants, animals, soil, water, atmosphere and extreme habitats [Spencer and Spencer, 1997]. Besides, being of fundamental scientific interest, an understanding of carbon nutrition and metabolism is essential for full exploitation of yeasts in biotechnology, not only for the production of useful products but also for the microbial treatment of carbon-containing waste and for an understanding of spoilage of natural and man-made materials [Walker, 1998]. In recent years, knowledge of yeast cell physiology has considerably lagged behind that of yeasts genetics and molecular biology. For example important aspects of the regulation of yeast growth and metabolism, which are crucial for successful exploitation of yeasts in industry, are still poorly understood from a fundamental point of view.

Knowledge on stress and stress responses is crucial to our understanding on how single-cell and multicellular organisms adapt to changing environmental and physiological conditions. *S. cerevisiae* is the best genetically and biochemically characterized eukaryote, and thus it has been widely used in studies on stress response [Hohmann, 1997], in spite of the fact that it is only moderately resistant to salt stress when compared with other microorganisms. Halotolerant yeasts can be highly salt resistant, meaning that, comparing with *S. cerevisiae,* they migth use very different salt stress mechanisms defences. *C. halophila* has revealed in a salt stress survey performed on 42 different yeast species to be highly resistant to salt stress, growing in media containing up to 4.5 M NaCl [Lages *et al*., 1999]. Considering the reasonings above we chose *C. halophila*, an extremely salt resistant yeast with importance in the production of soy-sauce, as a good candidate to unveil some of the physiologicals mechanisms underlying extreme salt tolerance

The main goals of this thesis were to accumulate basic knowledge about stress responses in a

non conventional highly resistant yeast: (i) for further comparison with the well documented responses of *S. cerevisiae*, (ii) to fullfill in part, the lack of physiological knowledge on salt stress response in highly resistance yeasts and (iii) to emphasize the importance of research using less known yeasts, highlighting and reinforcing their possible biotechnological applications.

This thesis work focused on studying the physiological and biochemical long-term response to salt stress of the yeast *C. halophila,* which has revealed to be extremely resistant to high sodium chloride concentrations [Lages *et al*., 1999]. The first part of the work (Chapter II) concerned the growth kinetics in the absence and in the presence of different salt concentrations at variable initial medium pHs. We also measured the growth kinetics with different carbon sources and looked for substrate simultaneous or sequential utilization. The next step involved the comprehension of the type of solutes produced and excreted in the absence or in the presence of salt, namely the definition of the osmolyte type used by *C. halophila*. In Chapter III the characterization of glucose, glycerol and mannitol transport systems is attempted, regarding the importance of the transmembrane transport of solutes in correct prosecution of the metabolism. Main metabolism regulation in the presence o salt is addressed in Chapter IV by measuring global fermentation and respiration fluxes as well as determining the specific activities from enzymes selected as important towards our understanding of internal metabolism under stress conditions. The assessment of ion intracellular concentrations in Chapter V closes the set of basic physiological and biochemical assays proposed for the beginning of small but significant knowledge about the long-term physiology of extremely salt tolerant yeasts in the presence of high salt concentrations.

To conclude, the results obtained in the chapters mentioned are brought in perspective in a general discussion in Chapter VI as well as the future perspectives opened by this work.