Chapter 4

General metabolism

4.1. *Introduction*

4.1.1 *Glycolysis*
4.1.2 *Metabolic alternatives of intracellular pyruvate*
4.1.3 *The tricarboxylic acid cycle (TCA)*
4.1.4 *Pentose phosphate pathway*
4.1.5 *Ethanol metabolism*
4.1.6 *Sugar alcohol metabolism*
4.1.6.1 *Glycerol metabolism*
4.1.6.1.1 *Glycerol synthesis*
4.1.6.1.2 *Glycerol utilization*
4.1.6.2 *Mannitol metabolism*
4.1.6.2.1 *Mannitol synthesis*
4.1.6.2.2 *Mannitol utilization*
4.1.7 *Futile cycles in fungi*
4.1.8 *Redox balance in the metabolism of yeasts*

4.2. *Results and discussion*

4.2.1. *Enzyme activities of main metabolic pathways*
4.2.2. *Enzyme activities of main metabolic pathways in salt-stress grown cells*
4.2.3. *Glycerol metabolic pathways in salt-stress grown cells*
4.2.4. *Mannitol metabolic pathways*
4.2.5. *Pyridine nucleotides balance under salt stress*

4.3. *Concluding remarks*

Summary

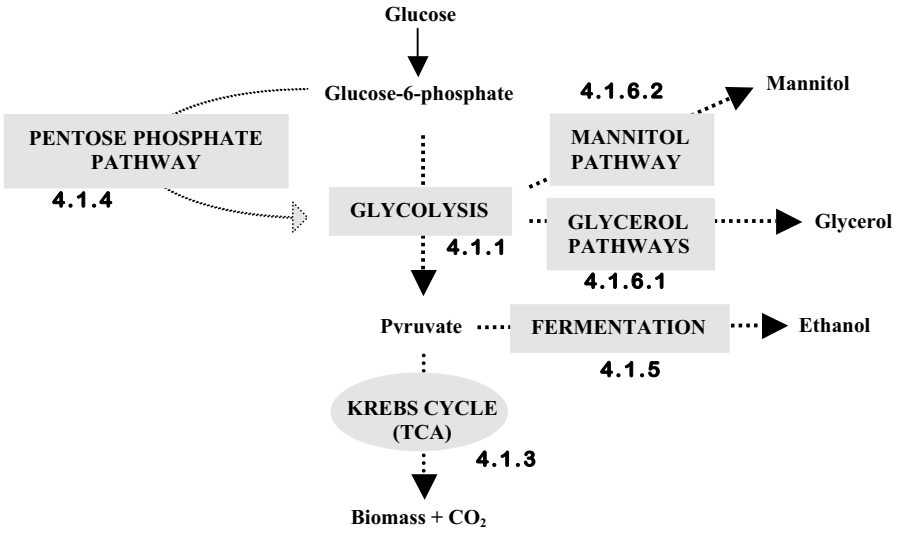
Yeast metabolism refers to the biochemical assimilation and dissimilation of nutrients by yeast cells. The subject therefore encompasses all enzymatic reactions within the yeast cell and how such reactions are regulated. This chapter presents biochemical results concerning the determination of specific activities of enzymes belonging to (i) main metabolic pathways such as glycolysis, pentose phosphate pathway and Krebs cycle, (ii) from glycerol metabolic pathways and (iii) from mannitol metabolic pathways. *C. halophila* revealed before to be an organism using essentially a respiro-fermentative metabolism, at least under the growth conditions used to assay the physiological characteristics exposed during this thesis. Consistent with this type of metabolism and with the high increase observed in CO_2 production in extreme salt stress presented in Chapter 2, we also detected increased ethanol dehydrogenase activities in stress conditions, being the increase observed of the same order of magnitude as those registered for CO_2 production. Furthermore, the glycolysis enzymes hexokinase and glyceraldehyde-3-phosphate dehydrogenase were also highly increased under stress, suggesting that glycolytic flux is enhanced under stress. Malate dehydrogenase and isocitrate dehydrogenase, considered here as part of the Krebs cycle, also showed to be increased under salt stress, confirming the results obtained before for an enhanced oxygen consumption under stress. Together these results point to a general pattern of enzyme activities stimulation under salt stress. Enzymes from the glycerol pathway revealed an increase in activity on cells grown in the presence of salt, being in accordance with its compatible solute function and their accumulation under salt stress. Enzymes from mannitol synthesis pathway were also increased under stress in spite of this compound do not revealed to be involved in *C. halophila* osmoregulation. Cofactors intracellular concentrations were also determined and evidenced a redox balancing under stress, contributing to the idea of *C. halophila* having an equilibrated metabolism under extreme salt stress thus emphasizing this yeast extremely halotolerant character.

4.1. Introduction

Yeasts are chemoorganotrophic microorganisms deriving their chemical energy, in the form of ATP, from the breakdown of organic compounds. However, there is metabolic diversity in the way in which yeasts generate and consume energy from carbon substrates. Knowledge of such regulation is crucial in yeast biotechnologies, which exploit carbon metabolism in the production of industrial commodities, but is also of importance in acquiring basic knowledge on spoilage and high resistant yeasts.

Carbohydrates are usually the major carbon sources taken in by fungi and these are metabolized to provide energy and precursors for the synthesis of cellular structures. Most yeasts use sugars as their preferred carbon and energy sources. The ways in which carbohydrates are translocated by yeasts from their growth environment into the cell were discussed in Chapter 3. The following sections consider the principal fates of carbohydrates in yeasts, namely: the dissimilatory pathways of fermentation and respiration according to Figure 4.1.

Figure 4.1. Main pathways approached in the following sections.



4.1.1. *Glycolysis*

The most common biochemical pathway used by fungi as a means of energy production from hexoses, namely glucose, is glycolysis, also termed Embden-Meyerhof-Parnas pathway for its major discoverers, and takes place in the cytoplasm. All the 800 yeast species described so far are using glycolysis for the oxidation of hexoses phosphate into pyruvate [Pronk *et al*., 1996].

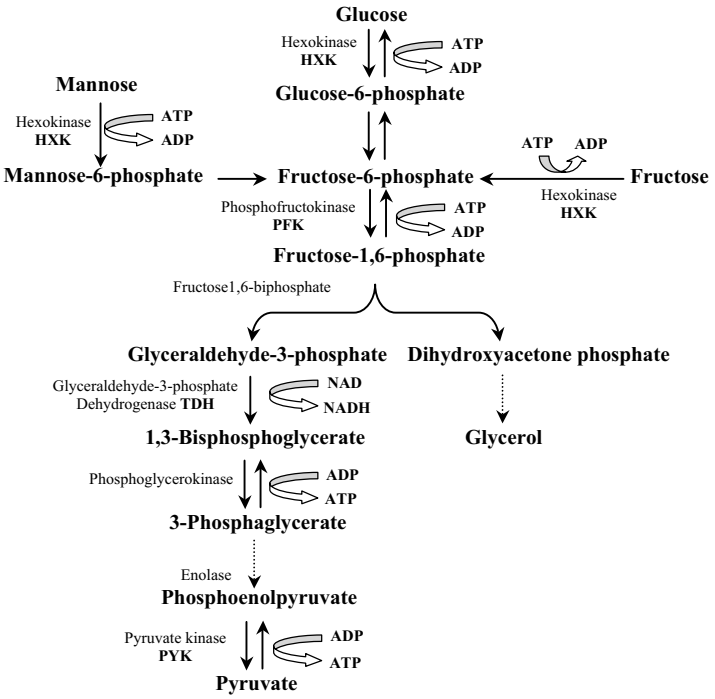
The first stage in glycolysis is a series of preparatory rearrangements, reactions that do not involve oxidation-reduction and do not release energy but lead to the production from glucose of two molecules of the key intermediate, glyceraldehyde 3-phosphate. In a second stage, oxidation-reduction

occurs being produced both ATP and two molecules of pyruvate. In a third stage pyruvate is subjected to a series of oxidation-reduction reactions and may originate by fermentation products such as ethanol, lactic acid, CO_2, or be channeled into Krebs cycle for respiration.

The intracellular hexoses enter the glycolytic pathway after a phosphorylation step, which in the case of glucose leads to glucose-6-phosphate. This molecule is a central key for diverse aspects of the cell, since it is the precursor both of the pentose phosphate pathway and for synthesis of oligo and polyssacharides. Usually, glucose, fructose and mannose are phosphorylated by hexokinases. The term hexokinase (HXK) is used to designate enzymes with broader hexose substrate specificity. HXKs are generally capable of phosphorylating glucose, fructose and mannose, but also other monosaccharides at lower rates. However, several times we are faced with designations such as glucokinases, fructokinases and so on, which refer to hexokinases with specificity for some particular hexose. For instance, glucokinase has a high specificity for glucose and very low, or non-existent, specificity for other monosaccharides. *S. cerevisiae* is known to possess two hexokinases and a glucokinase, encoded by *HXK1*, *HXK2* and *GLK1*, respectively [Lobo and Maitra, 1977]. Hexokinases were also found in several other yeasts such as *K. lactis* (only one hexokinase) [Prior *et al*., 1993], *C. tropicalis* (two hexokinases and a glucokinase) [Hirai *et al*., 1977], *Y. lipolytica* (one hexokinase and one glucokinase) [Petit and Gancedo, 1999] and *S. pombe* (two hexokinases) [Petit *et al*., 1996]. Most hexokinases are inhibited by trehalose-6-phosphate, a compound that plays an important role in the regulation of glycolysis in *S. cerevisiae* [Blázquez *et al*., 1993]. The hexokinase of *Y. lipolytica* shows the strongest inhibition by trehalose-6-phosphate yet found [Petit and Gancedo, 1999]. But hexokinases from *S. pombe* are peculiar with regard to the inhibition by treahalose-6-phosphate, being apparently regulated in a different way from those of *S. cerevisiae*. Hexokinase II is known in *S. cerevisiae* for its role in catabolite repression of certain genes [Gancedo, 1998], but the information relative to other yeasts is scanty. Glucose-6-phosphate is, after being produced, converted to its isomeric form, fructose-6-phosphate, and a second phosphorylation leads to the production of fructose 1,6-bisphosphate, which is a key intermediate product of glycolysis and is catalyzed by an irreversible phosphofructokinase. This enzyme and the irreversible pyruvate kinase are considered key regulatory enzymes in glycolysis, whose activity is in yeasts influenced by numerous effectors, including AMP/ATP ratios, *i.e*., internal P_i availability, ammonium ions and fructose 2,6-biphosphate [Gancedo and Serrano, 1989]. Another very important step in glycolysis is the conversion of glyceraldehyde-3-phosphate into 1,3-bisphophoglycerate catalyzed by glyceraldehyde-3-phosphate dehydrogenase. In this step, oxidation of the aldehyde generates an energy-rich acyl phosphate bond. Furthermore, NADH is generated and must be reoxidized for glycolysis to proceed and to maintain redox balance. In *S. cerevisiae*, three genes named *TDH1-3* encode three different isoenzymes, being the *TDH3* the most representative in the cell [McAlister and Holland, 1985]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been considered a classical cytosolic glycolytic protein. However, recent evidence demonstrates that mammalian GAPDH displays diverse activities that are unrelated to glycolysis in different subcellular locations. In yeasts such as *K. marxianus* [Fernandes *et al*., 1995], *C. albicans* [Villamon *et al*., 1999] and more recently in *S. cerevisiae* [Delgado *et al*., 2001], GAPDH has been found on the cell surface, where it may have different roles. Expression of the three GAPDH in *S. cerevisiae* is not coordinately regulated in cells subjected to stress from glucose depletion or heat shock, being the expression of *TDH1* restricted to stressed cells [Boucherié *et al*., 1995]. *TDH1* gene product, but not *TDH2-3*, was later confirmed by Norbeck and Blomberg (1996, 1997b) to be responsive to salt stress, thus pointing to a role of this protein in general stress response. GAPDH is also an important enzyme in yeast metabolism because

it is the first catalytic step after the bifurcation in glycolysis (Figure 4.2). GAPDH activity is believed to be of importance especially in stress conditions, where glycolytic flux may be strongly deviated to glycerol accumulation through dihydroxyacetone phosphate, being the processes of fermentation or respiration compromised by the efficiency of GAPDH. Nevertheless, as said above, at least one of the isoenzymes is stress responsive [Norbeck and Blomberg, 1996,1997b], confirming the necessity of glycolysis prosecution under stress.

Figure 4.2. Glycolysis general scheme for three main sugars in yeasts. The sequence of enzymatic reactions in the conversion of glucose to pyruvate.



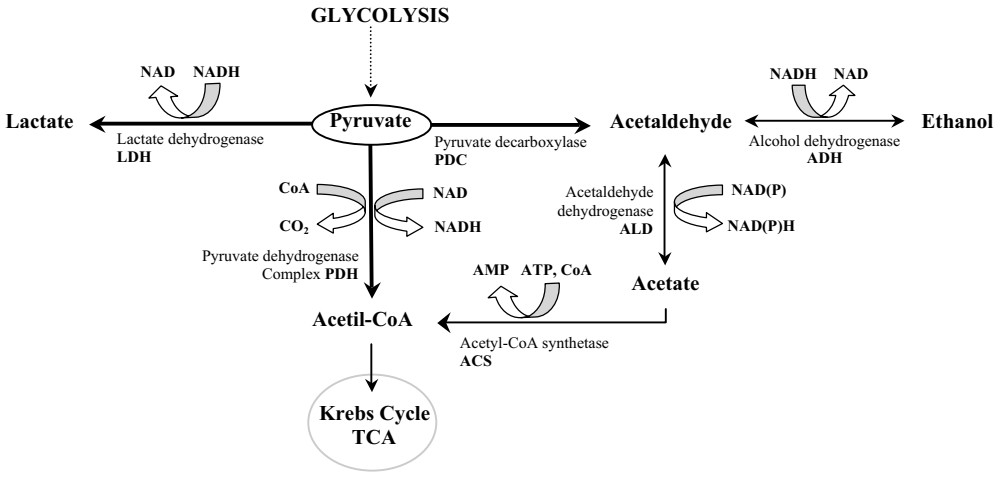
In glycolysis, two ATP molecules are consumed in the two phosphorylations of glucose, and four ATP molecules are synthesized from each 1,3-bisphosphoglyceric acid converted to pyruvate. Thus, the net gain to the organisms is two molecules of ATP per glucose fermented. Glycolysis leads also to the production of two NADH which will be re-oxidized in the reduction of pyruvate (the organic fermentation product).

4.1.2. *Metabolic alternatives of intracellular pyruvate*

Yeasts do not form a homogeneous group as far as energy-yielding metabolism is concerned. Glycolysis can take place under aerobic as well as anaerobic conditions and the fate of pyruvate, a

glycolytic intermediate and a ramification point, will determine the type of energy metabolism that is being used. The two major fates of the pyruvate produced in glycolysis are either its oxidation to CO_2 or its transformation to ethanol or other compounds through oxidoreductive metabolism (Figure 4.3). In most yeast under aerobic conditions, oxidation will be predominant, while transformation to ethanol takes place in anaerobic conditions or at high glucose concentrations even in aerobic conditions in those yeasts that present a Crabtree effect such as *S. cerevisiae*. When the main end-product of glycolysis, pyruvate, cannot be completely degraded to CO_2 via TCA cycle, it enters one of two fermentation pathways, lactic or alcoholic. In lactic fermentation, pyruvate is transformed directly to lactate by a lactate dehydrogenase, a process that can occur in the latter stages of wine fermentation. Nevertheless, the most important fermentation pathway occurring in yeasts is the alcoholic fermentation. Pyruvate obtained through this process is decarboxylated to acetaldehyde via pyruvate decarboxylase; the acetaldehyde in then reduced to ethanol via alcohol dehydrogenase. Pyruvate decarboxylase is encoded by *PDC1-5* genes in *S. cerevisiae*, being some of them essential to grow on glucose [Hohmann, 1997b]. On the contrary, *K. lactis* has only one gene assigned, *RAG6* [Bianchi *et al*., 1996], but is not essential for growth on glucose, which highlights the different lifestyles of these two yeasts (in aerobic conditions *S. cerevisiae* is an aerobic fermentative yeast while *K. lactis* is an aerobic respiratory yeast).

Figure 4.3. Metabolic fates of pyruvate in yeasts.

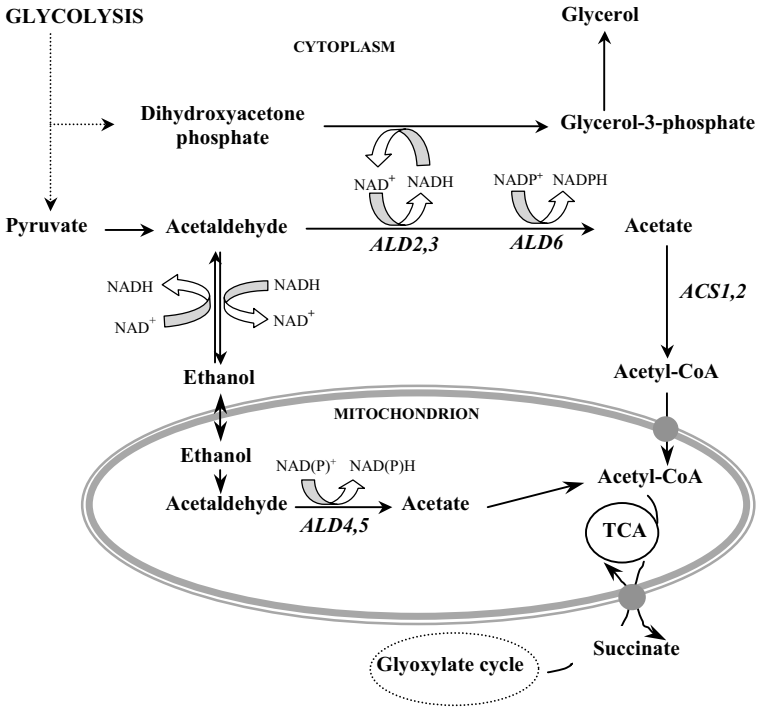


Acetic acid is also a by-product of the metabolism of certain yeasts, being synthesized from acetaldehyde in the presence of acetaldehyde dehydrogenase (Figure 4.3). Simultaneous production of ethanol and acetate is frequent and is regulated by the expression and affinity of acetaldehyde for each enzyme [Wang *et al*., 1998]. Ethanol production is the final step in the alcoholic fermentation process and leads to a neutral redox balance, since it is re-oxidizing the NADH formed during the first steps of

glycolysis (Figure 4.2 and 4.3). On the contrary, acetate formation during a fermentation process may lead to a surplus of reduced cofactors NAD(P)H (Figure 4.3).

Aldehyde dehydrogenases are important enzymes not only for the metabolism of acetaldehyde produced from ethanol, but also because of toxic aldehydes produced in some stress situations [Llorente and Castro, 1977]. In humans, metabolic disorders and clinical problems like for instance alcoholism and carcinogenesis [Lindahl, 1992] have been associated with mutations in genes encoding these enzymes. Information about the number of this type of enzymes in *S. cerevisiae* and the corresponding genes has been confused for some time. This led Navarro-Aviño and collaborators (1999) to propose a novel nomenclature for aldehyde dehydrogenases. They reported the existence of five ALDH, which corresponding genes names proposed were *ALD2-6*. Ald2p and Ald3p are cytosolic enzymes, stress-induced and glucose repressed, using NAD^+ as co-enzyme. Ald4p and Ald5p are mitochondrial enzymes, activated by K^+, being Ald5p specific for $NADP^+$ and constitutive [Wang *et al*., 1998], and Ald4p glucose-repressed using both NAD^+ and $NADP^+$ [Jacobson and Bernofsky, 1974]. Ald6p was assigned to be cytosolic, activated by Mg^{2+}, constitutive and with specificity for $NADP^+$ [Dickinson, 1996; Meaden *et al*., 1997].

Figure 4.4. Metabolism of acetic acid in *S. cerevisiae*.



The two major yeasts ALDH correspond to the mitochondrial Ald4p and the cytoplasmic Ald6p. Ald2p and Ald3p have STRE elements in their gene promoter and are induced by several types of stress such as heat shock, oxidative stress and DNA damage, in a Hog1-independent manner [Akhtar

et al., 1997]. *ALD6* gene is also transiently induced by osmotic shock but in a HOG-dependent manner [Akhtar *et al*., 1997]. The characterization of Ald3p as NAD^+-dependent acetaldehyde dehydrogenase and the osmotic induction of *ALD3* gene suggested that this enzyme could play a role in cytoplasmic redox balance during osmo-induced glycerol biosynthesis (Figure 4.4.). Thus the NADH consumed during dihydroxyacetone phosphate reduction to glycerol, could be provided by the NAD^+-dependent oxidation of either acetaldehyde or 2-oxoglutarate, because glycerol production is accompanied by the production of acetic and succinic acids [Gancedo and Serrano 1989]. However, the increase in ALDH activity is dispensable for glycerol production and osmotic adjustment. Thus, apparently redox balance could still be accomplished by mitochondrial production of either succinic or acetic acid. Furthermore, *ALD2,3* genes, in a similar way as proposed before for *ALD6* by Meaden and collaborators (1997), revealed to be very important in ethanol utilization as carbon source, possibly contributing to cytosolic acetyl-CoA synthesis during growth in ethanol [Navarro-Aviño *et al*., 1999]. Mitochondrial Ald4p has been reported to be present in ethanol grown cells (the gene is glucose repressed), and thus it would permit the mitochondria to utilize acetaldehyde for the production of energy and the generation of reducing equivalents (NADPH) for intramitochondrial biosynthetic reactions [Jacobson and Bernofsky, 1974]. Aldehyde dehydrogenases are responsible for the small amount of acetate produced during glucose fermentation [Gancedo and Serrano, 1989]. Acetate production is increased at alkaline pH values, and this can be explained by the pH optimum of the aldehyde dehydrogenase [Neuberg, 1946; Tamaki and Hama, 1982]. This enzyme should be particularly active in members of the genus *Brettanomyces* and in the yeast *Z. acidifiens* (*Z. bailii*) all of which produce large amounts of acetic acid during fermentation [Gancedo and Serrano, 1989]. As far as we know, there is no information on aldehyde dehydrogenases in yeasts other than *S. cerevisiae* nor even in those yeasts which produce visible amounts of acetic acid, except for *C. tropicalis* and *C. lipolytica* growing in n-alkanes [Yamada *et al.,* 1980]. For these yeasts it was reported aldehyde dehydrogenase activities in peroxisomes and mitochondria being involved in lipid synthesis in those cellular compartments [Yamada *et al*., 1980].

Frequently, under aerobic conditions, oxidation of pyruvate to CO_2 occurs via the TCA cycle. Nevertheless, pyruvate may be used simultaneously to produce ethanol (fermented) and oxidized through the Krebs cycle (respirated), being this type of metabolism termed respiro-fermentative. Pyruvate enters the TCA cycle undergoing a decarboxylation catalyzed by a pyruvate dehydrogenase complex, a mitochondrial multienzyme complex that is formed by three different components (Figure 4.3). The localization of this complex at mitochondria implies that cytosolic pyruvate must be transported into this organelle. Acetyl-CoA can also be formed in the cytosol through the so-called pyruvate bypass that involves the synthesis of acetyl-CoA through the concerted action of pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl CoA synthetase (Figure 4.3). These reactions followed by transport of the formed acetyl CoA to the mitochondria could in principle "by-pass" the action of pyruvate dehydrogenase complex, when this is repressed. Acetyl-CoA is a central molecule in yeast metabolism due to the involvement in anabolic processes such as fatty acids, sterols and amino acids synthesis. This is corroborated by the fact that a *S. cerevisiae* strain deleted in both genes, *ACS1* and *ACS2*, coding for acetyl CoA synthetase does not grow in media containing the most simple and common carbon sources [van den Berg *et al*., 1996; van den Berg, 1997]. The two forms of acetyl-CoA synthetase evidence differences in regulation, localization and kinetic aspects suggesting different roles in metabolism. Strains disrupted in *ACS2* gene grew normally on ethanol and acetate, but were enable to grow on glucose, since *ACS1* is subjected to glucose catabolite inactivation [de Jong-Gubbels *et*

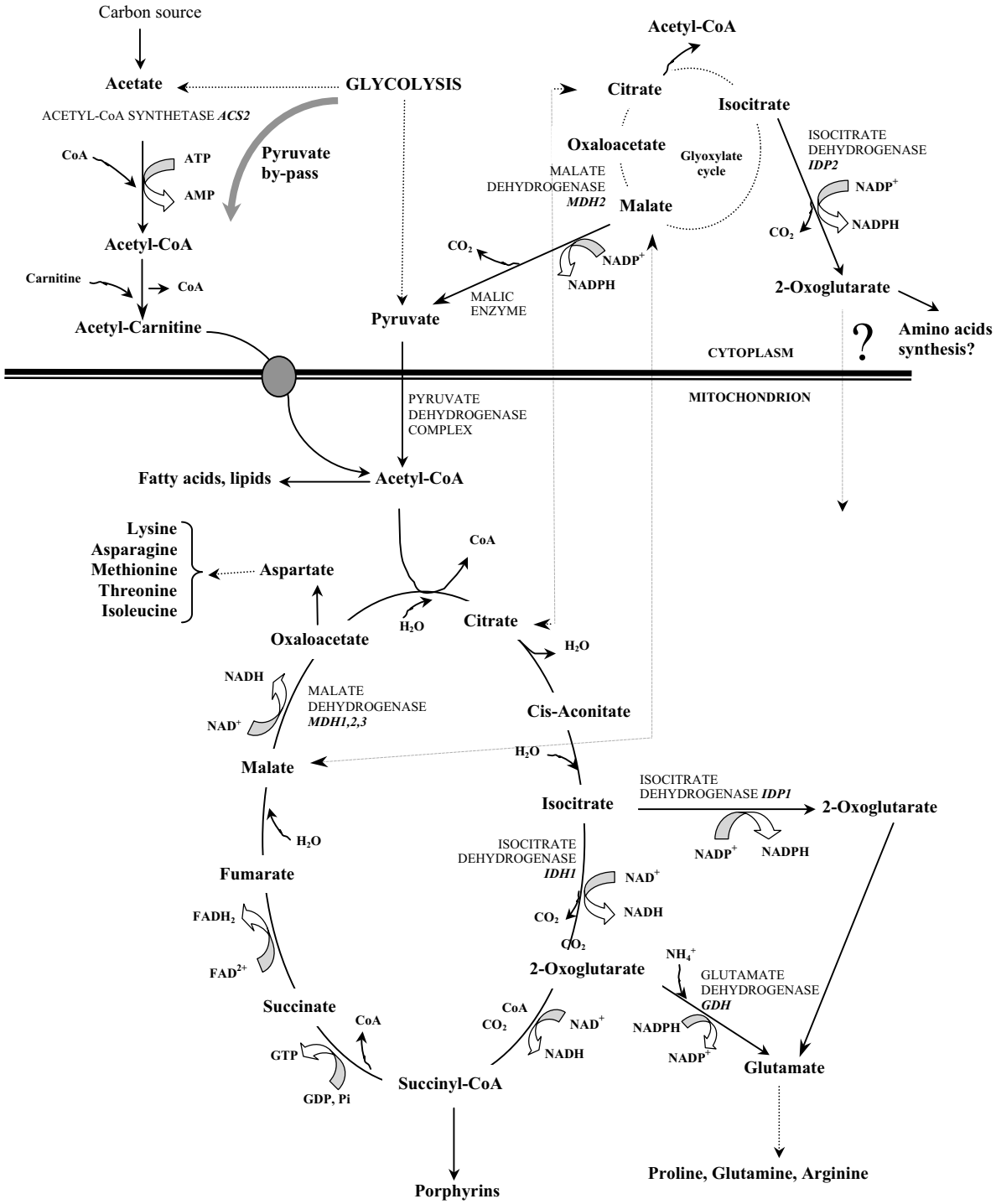
al., 1997]. In *S. cerevisiae*, exposure of cultures to high sugar concentrations results in metabolism occurring essentially via pyruvate decarboxylase, and under these conditions acetate is formed as a by-product, indicating that the *in vivo* activity of acetaldehyde dehydrogenase exceeds that of acetyl-CoA synthetase [van den Berg *et al*., 1996], although it is not the sole cause of acetate accumulation in *S. cerevisiae* [de Jong-Gubbels *et al*., 1998].

4.1.3. *Tricarboxylic acid cycle (TCA)*

Due to its dimension, TCA cycle, also termed Krebs cycle or citric acid cycle, will not be presented extensively, and only the most important reactions concerning the thesis subject will be approached. The TCA cycle is carefully regulated to ensure that its level of activity corresponds closely to cellular needs. TCA cycle, like glycolysis is an amphibolic pathway since it fulfils both catabolic and anabolic functions. In fact TCA cycle provides several intermediates for various biosynthetic processes, such as amino acids synthesis and thus the presence of anaplerotic pathways that replenish the cycle with intermediates is indispensable to keep it functioning (Figure 4.5). Two important anaplerotic pathways in yeasts are the glyoxylate cycle (Figure 4.5) and the pyruvate carboxylase, a cytoplasmic enzyme that catalyzes the conversion of pyruvate into oxaloacetate. In yeasts growing in a minimal medium with a sugar as carbon source and NH_4^+ as nitrogen source, pyruvate carboxylase is the main anaplerotic reaction [Flores *et al*., 2000]. Glyoxylate cycle is required for growth in minimal medium in carbon sources with less than three carbon atoms, such as ethanol or acetate. *S. pombe* lacks the enzymes of the glyoxylate cycle and this could explain the lack of growth in ethanol of this yeast [Flury *et al*., 1974].

In its primary role as a means of oxidizing acetyl groups to CO_2 and water, the TCA cycle is sensitive both to the availability of its substrate, acetyl-CoA, and to the accumulated levels of its principal end products, NADH and ATP. Actually, the ratio NADH/NAD^+ and the energy charge or the ATP/ADP ratio is more important than the individual concentrations. Other regulatory parameters to which the TCA cycle is sensitive include the ratios of acetyl-CoA to free CoA, acetyl-CoA to succinyl-CoA, and citrate to oxaloacetate. The major known sites for regulation of TCA cycle include two enzymes outside the Cycle, pyruvate dehydrogenase and pyruvate carboxylase, and three enzymes inside the TCA cycle, citrate synthase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase (Figure 4.5). Each of these sites of regulation represents an important metabolic branchpoint. Acetyl CoA generated either by pyruvate dehydrogenase complex or by the pyruvate bypass is the link between glycolysis and the TCA cycle (Figure 4.5). Acetyl-CoA reacts with oxaloacetate in a reaction catalyzed by citrate synthase to produce citrate (Figure 4.5). Since the equilibrium constant for the conversion of citrate to isocitrate is small these two intermediates make up a metabolic pool [Zubay, 1993]. The next possible regulatory site is the conversion of isocitrate to 2-oxoglutarate. Isocitrate is one of the intermediate compounds of the TCA cycle and is converted to 2-oxoglutarate (α-ketoglutarate) by an isocitrate dehydrogenase (Figure 4.5), being this enzyme highly regulated and a limiting step of the Krebs cycle [Zhao and McAlister-Henn, 1996]. In yeasts, the activity of isocitrate dehydrogenase is positively regulated by NAD^+ and AMP and to a less extent by isocitrate concentration, and is negatively regulated by NADH making it very sensitive to the NADH/NAD^+ ratio [Zubay, 1993]. In *S. cerevisiae* a mitochondrial NAD^+-dependent isocitrate dehydrogenase, whose two different subunits are encoded by the genes *IDH1* and *IDH2* [Cupp and McAlister, 1991; 1992], participates in the TCA cycle [Zhao and McAlister-Henn, 1996].

Figure 4.5. The tricarboxylic acid cycle (TCA) in yeasts. Some enzymes identified in *S. cerevisiae* and the corresponding genes are represented.



Furthermore, three differently compartmentalized $NADP^+$-dependent isocitrate dehydrogenases, encoded by the genes *IDP1*, *IDP2* and *IDP3* exist, and at least some of them are important in the production of NADPH [Minard *et al.*, 1998]. Idp1p is $NADP^+$-dependent and is localized in the mitochondria, where it seems to be the main source of NADPH in the mitochondria. Nevertheless, no relation with either Krebs cycle or the respiratory capacity of the mitochondria was found [Haselback and McAlister-Henn, 1993]. Idp2p, also $NADP^+$-dependent, is localized in the cytoplasm and seems to be associated with the production of 2-oxoglutarate, that after a conversion to glutamate may enter the mitochondria [Haselback and McAlister-Henn, 1993; Loftus *et al*., 1994]. All the isoenzymatic forms of isocitrate dehydrogenase are expressed at high levels in media with gluconeogenic carbon sources, although in glucose medium, the mitochondrial $NADP^+$-dependent form Idp1p predominates, which is probably linked to the synthesis of some amino acids derived from glutamate, which in turn results from the conversion of 2-oxoglutarate into glutamate through the action of a glutamate dehydrogenase encoded by *GDH* gene [DeLuna *et al*., 20001] (Figure 4.5). The reason why isocitrate dehydrogenase is so intensely regulated can only be understood if we consider reactions beyond the TCA cycle. Of the two compounds citrate and isocitrate, only citrate is permeable to the barrier imposed by the mitochondrial membrane. Citrate, which passes from the mitochondrion to the cytoplasm, plays a major role in biosynthesis because of its immediate regulatory properties. In the cytosol, citrate may undergo a cleavage reaction in which acetyl-CoA is produced (Figure 4.5). The acetyl-CoA produced in the cytosol from citrate breakdown is used in a number of biosynthetic reactions including the synthesis of lipids, some amino acids, vitamins, cofactors, and pigments. Malate is produced in the cytosol through the glyoxylate cycle and can return to the mitochondrion by diffusion, or converted in the cytosol to pyruvate through the action of the malic enzyme, resulting in the reduction of $NADP^+$ (Figure 4.5). The pyruvate is either utilized directly in biosynthetic processes, or like malate, can return to the mitochondrion by diffusion (Figure 4.5). In addition to its importance in providing cytosolic acetyl-CoA and NADPH, citrate also serves as a major regulator of the rate of fatty acid synthesis. The effect of small molecule modifiers on isocitrate dehydrogenase is appropriate in that an excess of citrate is an indication of a high-energy charge. As a result, conditions that favor inhibition of isocitrate dehydrogenase will favor an accumulation of mitochondrial citrate leading to an increased diffusion rate of citrate from the mitochondrion to the cytosol where the citrate can exert its multiple effects on biosynthesis and on glycolysis regulation.

Oxoglutarate is also a branch point metabolite, since it can be transaminated to form glutamate. Glutamate is needed in protein synthesis directly, and also is a precursor of a number of other amino acids (Figure 4.5). Thus, it is consumed in the cytosol in rather large amounts in a cell that is synthesizing protein rapidly. Therefore we would expect that the reaction catalyzed by oxoglutarate dehydrogenase would be regulated so as to retain carbon in the cycle when energy is in short supply, and to allow the concentration of 2-oxoglutarate to rise, facilitating its transamination to glutamate and exit from the cycle when the energy supply is high [Zubay, 1993]. Krebs cycle is completed with the reaction that transforms malate into oxaloacetate and is catalized by malate dehydrogenase (Figure 4.5). This enzyme also completes the anaplerotic glyoxylate cycle that converts isocitrate into malate. Three isoenzymes encoded by *MDH1*, *MDH2* and *MDH3* genes associated with mitochondria, cytoplasm and peroxisome, respectively, were described [McAlister-Henn *et al*., 1995]. Each of the isoenzymatic forms seems to have a specific function in metabolism. Strains interrupted in *MDH1* gene are incapable to grow on acetate as carbon and energy source, while *MDH2* interruption provokes incapability to grow on synthetic media with ethanol or acetate, although they may grow in

rich medium, suggesting auxotrofic deficiencies. *MDH3* interruption does not allow growth with oleic acid as carbon and energy source, indicating that this gene product is involved in peroxisomal beta-oxidation. It should be emphasized that besides playing a key role in catabolic reactions, the citric acid cycle is important to the cell for biosynthetic reasons as well. This is because the cycle is composed of a number of key carbon skeletons that can be drawn off for biosynthetic purposes when needed (Figure 4.5). Particularly important in this regard are the intermediates 2-oxoglutarate and oxaloacetate, which are precursors of a number of amino acids, and succinyl-CoA, needed to form the porhyrin ring of the cytochromes and several tetrapyrrole compounds (Figure 4.5.). Oxaloacetate is also important because it can be converted to phosphoenolpyruvate, a precursor of glucose in gluconeogenesis. In addition to these, acetyl-CoA provides the starting material for fatty acid biosynthesis [Madigan *et al.*, 1997].

Glycolysis and Krebs cycle encompasses oxidation reactions that produce a small amount of ATP by substrate-level phosphorylation and reduced equivalents such as NADH and $FADH_2$. These are later processed by electron transport to oxygen as terminal electron acceptor to yield a considerable amount of ATP. Although aerobic respiration produces higher amounts of ATP than fermentation, the efficiency is only of 43%, since part of the energy generated is lost as heat, while in fermentation efficiency is about 32%, which means that fermentation is a conversion process thermodynamically reasonably efficient [Madigan *et al*, 1997].

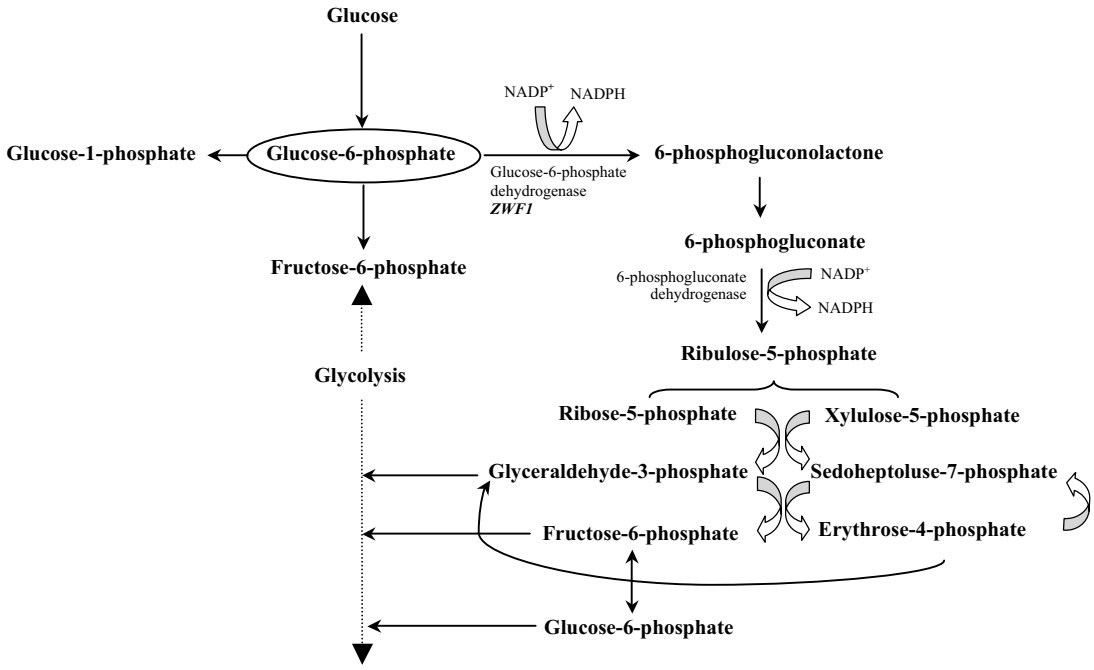
4.1.4. *Pentose phosphate pathway*

The pentose phosphate pathway, also called hexose monophosphate shunt or oxidation pathway is an important metabolic route implicated in the production of cytosolic NADPH for biosynthetic reactions and of ribose 5-phosphate for synthesis of nucleic acid and nucleotide cofactors; it also provides erythrose 4-phosphate for the synthesis of aromatic amino acids. It is generally accepted that pentose phosphate pathway is the major supplier of NADPH, and this is supported by the fact that the flux of glucose-6-phosphate through it is slower in cells cultivated in rich media [Gancedo and Serrano, 1989]. Under these conditions, the large amounts of NADPH required in minimal medium for biosynthesis of amino acids may be dispensable. Pentose phosphate pathway can be considered as a side branch of glycolysis, occurring in the cytoplasm, beginning with glucose-6-phosphate and re-entering at glyceraldehyde-3-phosphate and fructose-6-phosphate (Figure 4.6). Indeed, channelling of glucose-6-phosphate through the glycolytic pathway or through the hexose monophosphate pathway is a central point in carbohydrate metabolism, being glucose-6-phosphate in microorganmisms a substrate for three competing enzymes: glucose-6-phosphate dehydrogenase, glucose phosphate isomerase and phosphoglucomutase (Figure 4.6).

Pentose phosphate pathway has two phases: the oxidative phase gives pentose phosphate, NADPH and CO_2 from glucose-6phosphate whereas the non-oxidative phase allows interconversion between a pool of phosphorylated sugars. The first reactions of the pathway are two physiologically irreversible oxidative reactions, while the other ones are non-oxidative and reversible. It has been observed that the nitrogen source of the medium influences the amount of sugar directed to the pentose phosphate pathway. In *S. cerevisiae*, growth in a medium supplemented with amino acids decreased the flux through the pentose phosphate pathway [Gancedo and Lagunas, 1973]. In those yeasts that use nitrate as nitrogen source, an increase of the carbon flux through the pentose phosphate pathway shall be expected due to the increased NADPH requirement caused by the operation of nitrate and nitrite reductase; indeed this has been found in *C. utilis* [Bruinenberg *et al*., 1983b]. Glucose-6-

phosphate dehydrogenase (G6PDH), encoded by *ZWF1* gene in *S. cerevisiae* [Thomas *et al*., 1991], directs glucose into the pentose phosphate pathway by catalysing the oxidation of glucose-6-phosphate to 6-phospho-delta-gluconolactone (Figure 4.6). $NADP^+$-dependent glucose-6-phosphate dehydrogenase has been purified from *S. pombe* [Tsai and Chen, 1998] and in *C. utilis* [Domagk and Chilla, 1975].

Figure 4.6. Simplified mechanism of the pentose phosphate pathway in yeasts.



There are few studies concerning the enzymes from pentose phosphate pathway in other yeasts. *K. lactis* revealed to be able to use glucose exclusively via pentose phosphate pathway, in contrast to the situation in *S. cerevisiae*, which is one more argument setting apart the metabolism of these two yeasts [Jacoby *et al*., 1993]. The importance of pentose phosphate pathway in generating NADPH, may be emphasized by studies presented by Gancedo and Gancedo (1971) several years ago, where they determined the activity levels of the key enzyme glucose-6-phosphate dehydrogenase in cells of fermenting and non-fermenting yeasts grown either on glucose or ethanol. These authors verified that, although the activities were higher in glucose grown cells, the differences were relatively small. Thus they concluded that in vivo activity of the G6PDH may not be controlled at the level of synthesis but modulated by the concentration of some metabolites acting as effectors.

There are few enzymatic studies reporting the differences between yeasts using different types of metabolism. For instance, Caubet and collaborators (1988) reported a comparative study of the glycolytic and hexose monophosphate pathways in *C. parapsilosis* and *S. cerevisiae*. According to these authors, *C. parapsilosis*, which lacks alcohol dehydrogenase but is classified as fermentative yeast, presented high activities for glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate

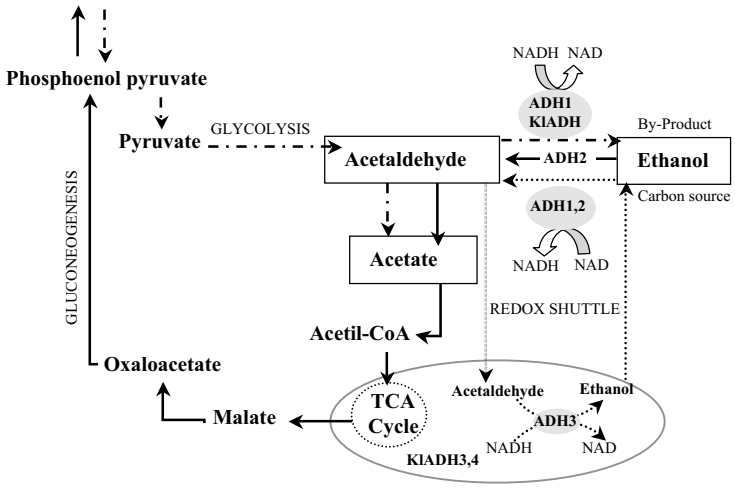
dehydrogenase and 6-phosphogluconate dehydrogenate, and a low sensitivity of fructose-1,6-bisphosphatase to catabolite repression, when compared with *S. cerevisiae*, which suggested that *C. parapsilosis*, as in other *Candida* species and opposite to *S. cerevisiae*, the glucose degradation occurred mainly through the hexose monophosphate pathway [Caubet *et al*., 1988].

More recently the transcriptional levels of the key enzyme G6PDH of the pentose phosphate pathway were reported to increase after a hyperosmotic shock with NaCl in *S. cerevisiae* and in *S. pombe* [Chen *et al*., 2003]. Nevertheless, there are not studies on long-term response to stress and thus the observed increase in G6PDH transcripts can be transient and not an effective, direct an important response in salt stressed cells. Indeed, deep enzymatic studies are missing not only concerning pentose phosphate pathway, but other pathways especially in non-conventional yeasts.

4.1.5. *Ethanol metabolism*

Ethanol may be used as carbon and energy source by most of the yeasts, but is widely known as a by-product of fermentation in fermentative and respiro-fermentative yeasts. Ethanol production during growth in sugars is more important in *S. cerevisiae* than in other yeasts. This is related to the type of predominant metabolism in relation to O_2 availability. *S. cerevisiae* ferments in aerobic conditions (Crabtree positive, Section 2.1.1). Central to the production or the utilization of ethanol are alcohol dehydrogenases, enzymes that catalyse the reversible reduction of acetaldehyde to ethanol (Figure 4.7).

Figure 4.7. General overview of ethanol metabolism in yeasts.



The kinetic and regulatory characteristics of these enzymes, the regulation of the transcription of the genes that encode them, or their subcellular localization determine the direction of the reaction catalysed under physiological conditions [Flores *et al*., 2000]. Utilization of ethanol implicates a first oxidation to acetaldedyde and a second oxidation to acetic acid catalysed by an aldehyde dehydrogenase (Figure 4.7). As said before, aldehyde dehydrogenases also participate in an

alternative pathway from pyruvate to acetyl-CoA when the pyruvate dehydrogenase complex is repressed. Ethanol is one of the most common gluconeogenic substrates for yeasts in nature. Yeasts have an ability to switch from the glycolytic mode in the presence of glucose to a gluconeogenic mode in its absence, or in the presence of substrates such as ethanol.

Alcohol dehydrogenases are implicated at least in three cellular events: (i) ethanol utilization as carbon source; (ii) production of ethanol as a end-product; and (iii) in the acetaldehyde-ethanol redox-shuttle (Figure 4.7). *S. cerevisiae* has at least two cytoplasmic alcohol dehydrogenase isoenzymes, encoded by *ADH1* and *ADH2*, and one mitochondrial isoenzyme, encoded by *ADH3* [Reid and Fewson, 1994; Leskovac *et al.*, 2002]. A fourth enzyme, Adh4p, was reported, but no specific characteristics have been assigned [Williamson and Paquin, 1987]. Adh1p is the constitutive form that is expressed during alcoholic fermentation and is induced by glucose, while Adh2p is repressed by glucose and is mainly involved in ethanol consumption. The mitochondrial form Adh3p as been recently reported to be involved in a redox shuttle in *S. cerevisiae* [Bakker *et al.*, 2000]. All these isoenzymes are NAD^+ dependent, with exception of a novel branched chain alcohol dehydrogenase $NADP^+$-dependent characterized by van Iersel and collaborators (1997) in *S. cerevisiae* var. *uvarum*. It was suggested that this form may have a role in flavor production during alcoholic fermentation, since this enzyme revealed a preference for long and branched-chain substrates with up to seven carbons atoms, in opposition to results reported for Adh1p to Adh4p. Another possible role assigned for this new enzyme was the maintenance of $NADP^+$/NADPH balance [van Iersel *et al.*, 1997]. Alcohol dehydrogenases have been through time isolated in other yeasts such as *S. pombe* [Russel and Hall, 1983], *K. lactis* [Saliola *et al.*, 1990, 1991], *C. albicans* [Bertram *et al.*, 1996], *P. stipitis* [Cho and Jeffries, 1998] and *Y. lipolytica* [Barth and Kunkel, 1979]. In the respiro-fermentative yeast *K. lactis* four enzymes were also characterized. KlAdh1p and KlAdh2p are cytosolic enzymes, NAD^+-dependent and preferentially expressed in glucose grown cells, while KlAdh3p and KlAdh4p are mitochondrial, may use either NAD^+ or $NADP^+$, being KlAdh3p repressed by ethanol and KlAdh4p induced by it [Saliola *et al.*, 1994]. *C. albicans* gene encoding alcohol dehydrogenase, *CaADH1,* was isolated and Northern analysis showed it to be less abundant in glucose and ethanol than in other carbon sources. In *P. stipitis* two cytoplasmic alcohol dehydrogenases have been identified, being PsAdh1p associated with growth under anaerobic conditions. Four alcohol dehydrogenases were also identified in *Y. lipolytica*, an NAD^+-dependent form that acts better on long chain alcohols, similarly to the one described in *S. cerevisiae* but $NADP^+$ dependent, and three $NADP^+$-dependent dehydrogenases.

4.1.6. *Sugar alcohol metabolism*

Sugar alcohols or polyols are widely distributed in fungi and since long date are being a matter of study [Barnett, 1968; Jennings, 1984], namely those used as compatible solutes. Osmotolerant yeasts capable of growing in high sugar or salt environments synthesize polyols such as glycerol functioning as compatible solutes. With regard to polyol metabolism, over 50% of yeasts surveyed can aerobically use glycerol, D-glucitol (sorbitol) and D-mannitol, while fewer than 10% of species utilized galactitol [Barnett, 1981]. Polyols may play very different physiological roles in microorganisms metabolism. As pointed out in Chapter 2, acyclic polyols have been frequently found as intracellular reserves in fungi, as compatible solutes, as scavengers and as redox balancing compounds. Among the yeasts, glycerol is the most common compatible solute and the most studied polyol, and thus the correspondent metabolism will be specifically detailed below.

4.1.6.1. *Glycerol metabolism*

A most surprising feature of glycerol is the number and variety of metabolic processes in which it is involved. Several of these pathways are similar in numerous organisms, demonstrating their fundamental importance and suggesting a common and ancient origin (Table 4.1). According to the Table 4.1, glycerol has proved to have high adaptative value in several species, both animal and plant [Brisson *et al*., 2001]. In spite of the importance of glycerol in various metabolic processes and, possibly, in the physiopathology of certain diseases, direct interest in this important molecule is still relatively recent and fairly modest [Brisson *et al*., 2001].

Table 4.1. Metabolic/physiological processes involving glycerol [Adapted from Brisson *et al*., 2001].

Bacteria	Yeast	Mouse	Human
●Carbon source	●Carbon source	●Gluconeogenesis	●Gluconeogenesis
●Energy production	●Energy production	●Energy production	●Energy production
●Osmoregulation	●Apoptosis	●Apoptosis	●Apoptosis
●Cryoprotection	● Redox potential balance	●Tumor formation	●Tumor formation
	●Recycling of inorganic phosphate	●Osmoregulation	●Osmoregulation
	●Osmoregulation		●Physical endurance
			●Glucose homeostasis
			● Cryoprotection

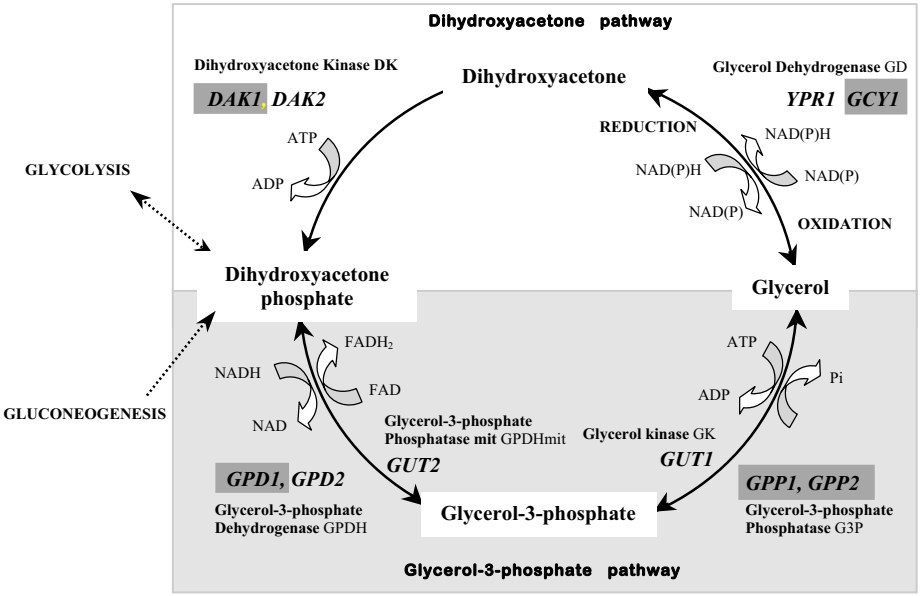
Glycerol metabolism has been particularly well studied in the yeast *S. cerevisiae* being involved in different aspects of their metabolism (Chapter 2). With regard to glycerol catabolism, this polyol is a non-fermentable carbon source, being utilized under aerobic conditions by many species of yeasts. On the other hand, glycerol is one of the major fermentation by-products when glucose or other easily fermentable sugars are converted to ethanol. However, the glycerol most studied aspect is the regulation of yeast metabolism associated with the wide function as compatible solute under osmotic/salt stress.

4.1.6.1.1. *Glycerol synthesis*

Glycerol is synthesized by reducing dihydroxyacetone phosphate (DHA) to glycerol-3-phosphate (G3P) catalysed by a NAD-dependent cytosolic glycerol-3-phosphate dehydrogenase (GPDH), followed by dephosphorylation of glycerol-3-phosphate (glycerol-3P) by a specific phosphatase (G3P) (Figure 4.8). Dihydroxyacetone phosphate can be provided by either glycolytic degradation of sugars or by gluconeogenic pathway when non-fermentable carbon sources are consumed. Isoenzymes of glycerol-3P dehydrogenase have been isolated, characterized and/or cloned in several eukaryotic organisms, from man [Hopkinson *et al.,* 1974] to *Drosophila melanogaster* [Bewley *et al*., 1989], unicellular algae [Gee *et al*., 1993] and yeasts. In the case of multicellular organisms, it has been shown that the GPDH isoenzymes expression is tissue and development dependent. In no instance has it been associated with stress response as in unicellular organisms. In green algae from the genus *Dunaliella* GPDH responds to salt stress and contributes to the intracellular accumulation of high amounts of glycerol [Avron, 1986]. Biochemical evidence showed the existence of three isoforms of GPDH in *D. tertiolecta* [Gee *et al*., 1993]. The most abundant form has been found in chloroplasts and

considered the osmoregulatory since its amount increased in cells subjected to stress [Gee *et al.*, 1993]. In yeasts, isoenzymes for GPDH have been primarily identified and studied in *S. cerevisiae*, in which case they were shown to be encoded by two isogenes, *GPD1* and *GPD2* [Larsson *et al.,* 1993; Eriksson *et al.,* 1995]. *GPD*-like genes have been identified in several other yeasts, *S. pombe* [Ohmiya *et al.,* 1995], *Z. rouxii* [Iwaki *et al*., 2001], *D. hansenii* [Nilsson and Adler, 1990; Thomé-Ortiz, 2004], *S. diastaticus* [Wang *et al.,* 1994] and *K. thermotolerans* and *P. angusta* [Neves *et al.,* 2004]. In *S. carlsbergensis* the enzyme was partially purified [Nader and Becker, 1994]. Differentiated regulation of the *GPD* isogenes has been extensively studied in *S. cerevisiae.* Gpd1p is essential for growth and is induced under osmotic stress in order to enhance the production of glycerol, the main osmoregulatory in *S. cerevisiae,* being its expression regulated by the high-osmolarity glycerol (HOG) response pathway [Blomberg and Adler, 1989; André *et al*., 1991; Larsson *et al*., 1993; Albertyn *et al*., 1994b]. Gpd2p is probably connected to cytoplasmic redox control, since it is necessary for normal growth and glycerol production under anaerobic conditions [Ansell *et al*., 1997; Björkqvist *et al*., 1997].

Figure 4.8. Glycerol metabolism in *S. cerevisiae.* Genes highlighted by a dark grey box have their expression increased under salt stress.



In *S. pombe*, as in *S. cerevisiae*, the two genes seem to have also distinct functions. One of them, *gpd1*$^{+}$ is transcribed in response to an osmotic upshift, being the target of a signalling pathway involving Wis1 Map Kinase [Aiba *et al*, 1995], while the other *gpd2*$^{+}$, is constitutively expressed at low levels [Ohmiya *et al*., 1995]. As *GPD1* in *S. cerevisiae*, *DAR1* expression in *S. diastaticus* is regulated by osmotic pressure, being the enzyme activity increased three fold in the presence of high osmolarity [Wang *et al*., 1994]. In *D. hansenii* GPDH as also been characterized as salt responsive, *i.e.*, specific

activities of a NAD^+-dependent GPDH increased after exposure to salt stress [Adler *et al.*, 1985; Nilsson and Adler, 1990; Thomé-Ortiz, 2004]. Up to the moment there are no evidences of the existence of a GPDH isoenzyme in this yeast. On the contrary, salt stress had a minimal effect on the level of *ZrGPD1* and *ZrGPD2* transcripts in *Z. rouxii* cells that had been subjected to salt stress for up to 12 h, a result suggesting that *ZrGPD1* and *ZrGPD2* are constitutively expressed. In fact, van Zyl and collaborators (1991) had already verified that the specific activity of GPDH in *Z. rouxii* was not affected by salt stress. Taking these results into account, Iwaki and collaborators (2001), as Ohshiro and Yagi (1996), suggested the existence in *Z. rouxii* of a glycerol assimilation pathway not involving GPDH proteins.

Genes encoding the specific phosphatases responsible for the dephosphorylation of the glycerol-3P have been identified in *S. cerevisiae*, and their gene product purified and characterized [Norbeck *et al.*, 1996]. Two isogenes *GPP1* and *GPP2 (HOR2)* corresponding to two isoenzymes were reported. (Figure 4.8) Both isoforms have a high specificity for DL-glycerol-3-phosphate [Norbeck *et al.*, 1996], and are required for glycerol biosynthesis. In analogy to *GPD* genes, *GPP* genes are differentially involved in the cellular responses to osmotic, anaerobic and oxidative stress [Påhlman *et al.*, 2001a]. Expression of both *GPP1* and *GPP2* is induced under hyperosmotic stress and this induction is partially dependent on the HOG pathway, although *GPP1* showed to be, in addition, under the strong influence of protein kinase A (PKA) activity [Påhlman *et al.*, 2001a]. The double mutant is hypersensitive to salt whereas the single mutants remain unaffected, indicating that *GPP1* and *GPP2* substitute well for each other. Furthermore, *GPP1* is transiently induced under anaerobic conditions [Påhlman *et al.*, 2001]. To our knowledge, no genes homologous to *GPP1/2* from *S. cerevisiae* have been described in other yeasts. The existence of isogenes differently regulated agrees with the multiplicity of roles attributed to glycerol and its related metabolic pathways.

Evidence of the existence of another pathway, via the intermediate dihydroxyacetone, originates from *S. pombe* [May and Sloan, 1981; Kong *et al.*, 1985; Gancedo *et al.*, 1986; Vasiliadis *et al.*, 1987] and *D. hansenii* [Adler *et al.*, 1985]. Dihydroxyacetone phosphate is dephosphorylated to dihydroxyacetone by a phosphatase and then reduced to glycerol by a glycerol dehydrogenase (GD) (Figure 4.8). No reports characterizing the phosphatase responsible for the dephosphorylation of dihydroxyacetone-P have been published. On the other hand, the enzymes able to catalize the same metabolic steps on the opposite direction, *i.e.*, dissimilating glycerol, glycerol dehydrogenase (GD) and dihydroxyacetone kinase (DK), have been shown to exist and were characterized and/or the genes cloned in *S. pombe* [May and Sloan, 1981; Kong *et al.*, 1985; Gancedo *et al.*, 1986; Vasiliadis *et al.*, 1987], in *D. hansenii* [Adler *et al.*, 1985], in *Z. rouxii* [van Zyl *et al.*, 1991] and in *S. cerevisiae* [Norbeck and Blomberg, 1996]. In this last case, GD and DK isogenes, respectively *GCY1*, *YPR1* and *DAK1, DAK2* (Figure 4.8), have been identified [Norbeck and Blomberg, 1996]. *GCY1* and *DAK1* have their expression increased under salt stress [Norbeck and Blomberg, 1996] (Figure 4.8). This pathway has been thought not to operating in *S. cerevisiae* being the glycerol 3-P pathway assigned as the only one operating for glycerol production [Ansell *et al.,* 1997]. Nevertheless, mutants lacking *GPD1* and *GPD2* have been shown to be able to produce glycerol under osmotic stress though to a considerable lesser extent than wild type starin [Oliveira *et al.,* 2003]. These mutants were shown to be able to grow in the presence of 1M NaCl after an extended lag phase overcoming the phenotype observed in plate assays. Anyway, for the time being, *GCY1* and *DAK1* although osmotically induced are only postulated to be associated with glycerol dissimilation via Dihydroxyacetone [Norbeck and Blomberg, 1997b]. Due to the poor expression of these genes under normal conditions there is in *S. cerevisiae*, to our knowledge, only one record of DK activity in stressed cells [Norbeck and Blomberg, 1997]. Two putative GD genes (*ZrGCY1 and ZrGCY2*) and a DHA

kinase putative gene (*ZrDAK1*) highly homologous to the ones of *S. cerevisiae* have been recently cloned in *Z. rouxii* [Iwaki *et al*., 2001; Wang *et al*., 2002]. In *Z. rouxii*, the specific activities of the NAD^+-dependent GD and the DK, which metabolise glycerol via DHA, increased nine- and four-fold respectively during osmotic stress when compared with non stressed conditions, while GPDH, the key enzyme in glycerol synthesis via G3P, did not increase in activity [van Zyl *et al*., 1991]. The same result was obtained with *ZrGCY1,2* mRNAs [Iwaki *et al*., 2001] upon incubation with NaCl, which corroborates that GD and DK are stimulated by salt. These results were further confirmed with the *ZrDAK1* gene expression in the *S. cerevisiae dak1*Δ*dak2*Δ strain, which do not produce significant effects on glycerol levels during osmotic stress. Synthesis of GD and DK are osmoregulated since the treatment of cells with cycloheximide in the presence of salt prevented the increase in their specific activity. This has also been found before in the regulation of GPDH in *S. cerevisiae* [Blomberg and Adler, 1989]. In spite of the results obtained so far in *Z. rouxii* concerning the enzymes of glycerol synthesis it is unknown whether GPDH and /or GD play a role in the salt tolerance, however results on these enzymes strongly suggest that they have a crucial role in maintaining osmotic balance across the plasma membrane under salt stress.

4.1.6.1.2. *Glycerol utilization*

Glycerol may be utilized as a carbon source under aerobic conditions by many yeast species [Gancedo and Serrano, 1989]. Some yeast, notably *C. utilis* [Gancedo *et al*., 1968] and *P. sorbitophila* [Lages *et al*., 1999], can grow on glycerol as readily as on glucose, and this ability was related with the presence in these yeasts of constitutive glycerol transporters [Lages *et al*., 1999]. Presently, there are two pathways well established for glycerol dissimilation in yeasts. These were presented above for glycerol synthesis: glycerol-3-phosphate pathway (phosphorylating pathway) and dihydroxyacetone pathway (oxidative pathway) (Figure 4.8).

Glycerol dissimilation through the glycerol-3-phosphate pathway has been reported on several yeast such as *C. utilis* [Gancedo *et al*, 1968], *S. cerevisiae* [Gancedo *et al*., 1968; Sprague and Cronan, 1977; Blomberg and Adler, 1989], *D. hansenii* [Adler *et al*, 1985] and *Z. rouxii* [van Zyl *et al*., 1991]. This pathway involves a primary phosphorylating step catalysed by a glycerol kinase (GK) which converts glycerol into glycerol-3-phosphate followed by an oxidation to dihydroxyacetone phosphate by a glycerol-3-phosphate dehydrogenase (G3PDHmit) localized in the mitochondria (Figure 4.8). Purification and characterization of GK was first attempted in *D. hansenii* by Nilsson and collaborators (1989). Glycerol kinase revealed to be constitutively expressed in glucose-grown cells [Adler *et al*, 1985], and highly specific for glycerol, indicating a specific role in glycerol metabolism [Nilsson *et al*., 1989]. Although GK specific activity in *S. cerevisiae* and in *C. utilis* were reported for the first time by Gancedo and collaborators in 1968, the GK gene, *GUT1* (**G**lycerol **UT**ilization) was only cloned and characterized in *S. cerevisiae* several years later by Pavlik and collaborators (1993). According to enzyme activity and transcripts analysis, synthesis of GK is repressed by glucose, and disruption of *GUT1* showed that this gene is required for growth in glycerol, but not in glucose or ethanol media [Pavlik *et al*., 1993]. Furthermore GK activity is highly reduced in media containing NaCl [Albertyn *et al*., 1994a]. Glycerol kinase specific activity was also measured in *Z. rouxii*, and similarly to *S. cerevisiae*, GK synthesis was repressed by glucose, being only detected in glycerol grown cells [van Zyl *et al*., 1991].

FAD^+-dependent glycerol-3-phosphate dehydrogenase mitochondrial catalizing the second step in glycerol dissimilation using the G3P phosphorylating pathway was detected in *S. cerevisiae*

[Albertyn *et al*., 1994a] and in *D. hansenii* [Adler *et al*., 1985], being its activity stimulated by osmotic stress. On the contrary, *Z. rouxii* also presented activity for this enzyme, but activity was reduced in the presence of salt [van Zyl *et al*., 1991]. The gene coding for G3PDHmit in *S. cerevisiae* (*GUT2*) has been cloned [Rønnow and Kielland-Brandt, 1993]. G3PDHmit is repressed by glucose and induced by glycerol according to its function in glycerol dissimilation.

Evidence for the existence of a second oxidative pathway involved in glycerol dissimilation was first reported in the green algae of the genus *Dunaliella* in which a glycerol dehydrogenase and a specific dihydroxyacetone kinase were described [Ben-Amotz and Avron, 1979; Lerner *et al*., 1980]. At the same time, specific activities of NAD^+ and $NADP^+$-dependent glycerol dehydrogenases were reported in several filamentous fungi [Jennings, 1984]. For instance, $NADP^+$-dependent glycerol dehydrogenase has been purified from *Mucor javanicus* [Dutler *et al*., 1977; Hochuli *et al*., 1977], *Neurospora crassa* [Viswanath-Reddy *et al*., 1978; Denor and Courtright, 1982], *Rhodotorula* sp. [Watson *et al*., 1969], *Aspergillus niger* [Schuurink *et al*., 1990], and more recently cloned the correspondent gene, *GLDB*, in *Aspergillus nidulans* [Fillinger *et al*., 2001; de Vries *et al*., 2003], whereas NAD^+-dependent glycerol dehydrogenase was detected in *Geotrichum candidum* [Itoh, 1982].

As mentioned above DHA oxidative pathway has been described and the respective enzymes characterized or cloned in some yeasts, being firstly described in *S. pombe* owing to the inexistence of an active glycerol kinase [May and Sloan, 1981]. This oxidative pathway is composed of a glycerol dehydrogenase (GH) that catalyzes the first oxidative step in which glycerol is transformed into dihydroxycetone, followed by a second phosphorylation step catalyzed by a specific dihydroxyacetone kinase (DK) (Figure 4.8). DK was purified and characterized in *D. tertiolecta* [Ghoshal *et al*., 2002], *P. pastoris* [Lüers *et al*., 1998], *A. nidulans* [Redkar *et al*., 1995] and cloned in *Z. rouxii* [Iwaki *et al*., 2001; Wang *et al*., 2002].

According to Vasiliadis and collaborators (1987) *S. pombe* and *S. malidevorans* were the only species of fission yeasts capable of growing on glycerol or dihydroxyacetone as carbon sources and both possessed GD and DK activities. Since these enzyme were subjected to catabolite repression, they were suggested to be the specifically involved in glycerol utilization as carbon source [May *et al*., 1982]. Babel and Hofmann (1982) have also demonstrated the presence of the same two enzymes in a number of other glycerol-utilizing yeasts. Furthermore, those yeasts which utilized both glycerol and methanol, none possessed GK activity. This is consistent with the fact that dihydroxyacetone occurs as a metabolite in the assimilation of methanol by yeasts [Hofmann and Babel, 1980; Lüers *et al*., 1998], being the phosphorylation of DHA an essential step in the utilization of methanol by yeasts [van Dijken *et al*., 1978; Babel and Loffhagen, 1979].

In the case of *Z. rouxii*, GD and DK were constitutively expressed and up-regulated by salt stress [van Zyl *et al*., 1991], while in *D. hansenii* although constitutively expressed their specific activity did not respond to osmotic stress [Adler *et al*., 1985]. As to cofactor utilization, GD in *D. hansenii* used NADP, whereas *Z. rouxii* used NADH. Glycerol dehydrogenase is an enzyme that catalyses the interconversion of glycerol and dihydroxyacetone, *i.e*., the oxidation of glycerol to dihydroxyacetone and the reduction of dihydroxyacetone to glycerol. It is however unknown if it uses the same cofactor in both directions or not, since each reaction is found to be implicated in different metabolic aspects in the cell. GD was assayed in *Z. rouxii* for the reduction of dihydroxyacetone, and in such case it used NADH. On contrary, *S. pombe* and *D. hansenii* were assayed for the oxidation of glycerol, (this is the most frequent type of assay) but revealed to use differently both cofactors. In *S. pombe* two forms of GD were reported, one is NAD^+ dependent while the other is $NADP^+$ dependent. The role of NAD^+

dependent GD in glycerol metabolism of *S. pombe* has been confirmed by the observation that mutants lacking NAD-GD were unable to grow on glycerol [Vasiliadis *et al*., 1987]. No clear function has been attributed so far for the $NADP^+$-dependent form. *D. hansenii* was shown to possess a $NADP^+$-dependent GD [Adler *et al*., 1985]. In the case of *Dunaliella* species, it was reported that GD used $NADP^+$ and NADPH as cofactors in the conversion between glycerol and dihydroxyacetone [Ben-Amotz and Avron, 1979; Lerner *et al*., 1980].

As mentioned before, glycerol dissimilation through the DHA was indicated to exist also in *S. cerevisiae* by the identification of the two putative isogenes for GD (*GCY1, YPR1*) and DK (*DAK1, DAK2*). Since deletion of either *GUT1* or *GUT2* confers inability to grow on glycerol, the physiological role of the alternative pathway during non-stressed conditions remains unclear. One of a number of possible roles for this pathway is to detoxify dihydroxyacetone by its conversion to DHP or to glycerol. In fact, it was recently published that dihydroxyacetone kinases are involved, in *S. cerevisiae* in detoxification of DHA [Molin *et al*., 2002], since *DAK1* gene was found to be upregulated under a large number of environmental conditions such as heat stress [Boy-Marcotte *et al*., 1999], osmotic stress by addition of external sorbitol or NaCl [Gash *et al*., 2000; Rep *et al*., 2000; Norbeck and Blomberg, 1997], hydrogen peroxide (oxidative stress) [Godon *et al*., 1998], cadmium stress [Vido *et al*., 2001] and starvation [Gash *et al*., 2000; Causton *et al*., 2001]. It is known that salt stress strongly aggravates DHA toxicity, independently of the carbon source, thus suggesting that detoxification of DHA might be a vital part of the physiological response during diverse stress conditions in many species [Molin *et al*., 2002]. Besides the detoxification properties attributed to the enzymes of the DHA pathway, it has been suggested that their osmotic induction might participate in an intrinsic regulatory mechanism for the fine tuning of turgor pressure when growing under osmotic stress, *i.e*., the excess of glycerol production through G3P being dissimilated through the DHA pathway, which should be energetically more favourable than glycerol efflux. Another alternative was later proposed which placed DHA kinase as a component in a ATP futile cycle activated under stress conditions [Blomberg, 2000].

4.1.6.2. *Mannitol metabolism*

As reviewed before in Chapter 2 mannitol is produced by several organisms and plays multiple functions. Nevertheless it is also used widely as carbon and energy source.

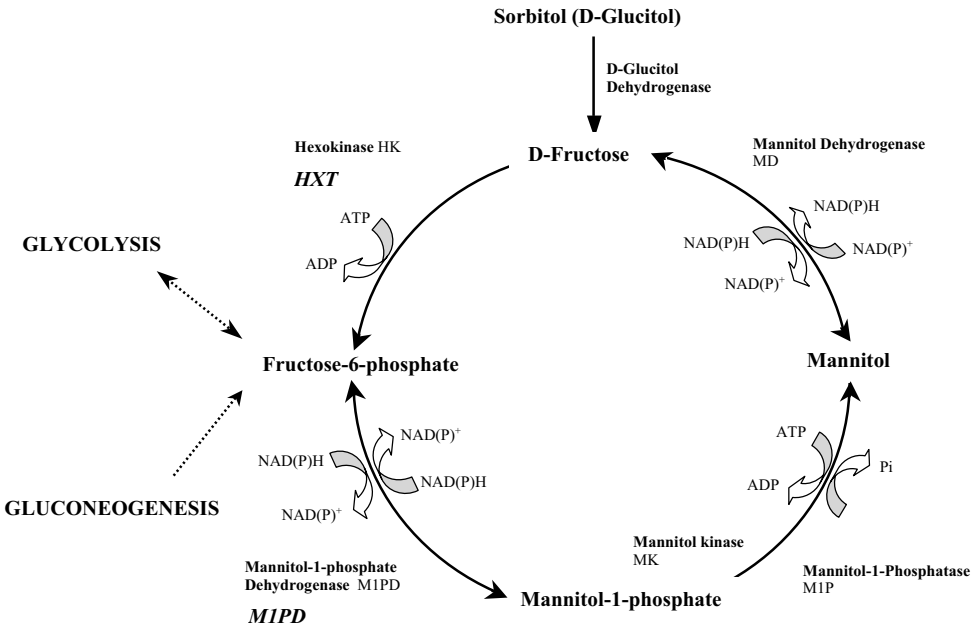
4.1.6.2.1. *Mannitol synthesis*

Mannitol has several important applications in food and pharmaceutical industry, and also in medicine where it is used to decrease cellular edema and increase urinary output [Von Weymarn, 2002]. For this reason, mannitol production has been studied from long date in good mannitol producing yeasts [Onishi and Suzuki, 1970]. Among the species belonging to *Torulopsis* genus, *T. anomala*, *T. apicola* [Zalashko *et al*., 1987], *T. bombicola* [Inoue and Kimura, 1986], *T. versatilis* and *T. mannitofaciens* were found to produce mannitol from simple carbons sources. From these, *T. mannitofaciens* was the one that possessed the highest rate of mannitol production [Onishi and Suzuki, 1971]. Some *Candida* species such as *C. petrovorus* [Iwamoto and Ozawa, 1973], *C. aliphatica* [Iwamoto *et al*., 1973], and *C. zeylanoides* [Hattori and Suzuki, 1974] have also been described as potential mannitol producers, being *C. lipolytica* shown to produce mannitol as the main sugar alcohol [De Zeeuw and Tynan, 1973]. In the case of *Rhodotorula minuta* mannitol was produced from various D-aldopentoses [Stankovic *et al*., 1989]. Several

other yeasts and filamentous fungi showed also to produce mannitol and were approached in Chapter 2. Another promising result concerning mannitol production in yeasts has been achieved with *Z. rouxii* [Looten *et al*., 1992].

Two pathways for mannitol biosynthesis in fungi were for the first time reported by Lewis and Smith (1967) (Figure 4.9). However, several studies concerning polyol metabolism in fungi had been published before that date in fungi such as in the basidiomycete *Schizophyllum commune* [Niederpruem *et al*., 1965], *Aspergillus oryzae* [Horikoshi *et al*., 1965], and in *Piricularia oryzae* [Yamada *et al*., 1961]. In one pathway, fructose is reduced directly to mannitol in the presence of NADH or NADPH by a mannitol oxidoreductase, which trivial name is mannitol dehydrogenase (MD). In the second pathway fructose-6-phosphate is reduced to mannitol-1-phosphate in the presence of NADH by a mannitol-1-phosphate oxidoreductase, which trivial name is mannitol-1-P dehydrogenase (M1PD). The mannitol-1-phosphate (M1-P) formed is hydrolysed by a mannitol-1-phosphatase to mannitol (M1P) (Figure 4.9).

Figure 4.9. Mannitol metabolism in fungi.



Mannitol metabolism has been studied mainly in fungi, namely with the determination, purification and characterization of the enzymes involved in the pathways mentioned above. In yeasts, the only study realized so far concerning mannitol metabolism was done in the opportunistic pathogen *C. neoformans* [Niehaus and Flynn, 1994; Suvarna *et al.*, 2000].

NAD^+-Mannitol-1-phosphate dehydrogenase (M1PD) has been studied in *Aspergillus*

candidus [Strandberg, 1969], *A. niger* [Kiser and Niehaus, 1981], *Aspergillus parasiticus* [Foreman and Niehaus, 1985], *P. oryzae* [Yamada *et al*., 1961], *Sclerotinia sclerotiorum* [Wang and Le Tourneau, 1972], *Penicillium* species [Boonsaeng *et al*., 1976], *Trichothecium* species [Hult *et al*., 1980], *Cladosporium* species [Hult *et al*., 1980], *Phycomycetes* species [Hult *et al*., 1980], *Mucor rouxii* [Boonsaeng *et al*., 1976], and in *Alternaria alternata* [Hult and Gatenbeck, 1978]. M1PD does not appear to be present in members of the Basidiomycotina [Hult *et al*., 1980]. This enzyme has been recently purified and characterized and the corresponding gene (*MPD1*) cloned and sequenced in the pathogenic basidiomycetous yeast *C. neoformans* [Suvarna *et al*., 2000]. M1PD appears to have high specificity for its substrates M1-P and fructose-6-phosphate (F6-P), but some activity was also found for glucitol-6-phosphate in *S. sclerotiorum* [Wang and Le Tourneau, 1972]. The equilibrium of the reaction involving the two substrates F6-P and M1-P favours in the direction of the latter, *i.e*., the direction of mannitol synthesis (Figure 4.9). *C. neoformans* M1PD reduced NAD^+ in the presence of M1P, sorbitol-6-phosphate and ethanol, and oxidized NADH in the presence of F6P, G6P and acetaldehyde. Besides, M1PD was absolutely specific for NAD^+ and NADH, since no reduction of NADP or oxidation of NADPH was observed [Survana *et al*., 2000]. In fact, up to date only the M1PD of the protozoon *Eimeria tenella* has been reported to use both NAD and NADP as cofactors [Schmatz *et al*., 1989; Schmatz, 1997]. An important feature of M1PD of *C. neoformans* is the fact that this enzyme was characterized as a zinc-containing long-chain alcohol/polyol dehydrogenase, and accepts ethanol and acetaldehyde as substrates. This may be related with the suggestion made by Boonsaeng and collaborators (1976), in which it was argued that the role of M1PD is in replenishment of the NAD^+ removed during glycolysis. Nevertheless without a deep study of the metabolism of a certain organism, namely the determination of specific activities during growth in different carbon sources, this suggestion is, at best, speculation.

Metabolism of mannitol in *Aspergillus* revealed in glucose grown cells the presence of a NAD^+ linked-M1P dehydrogenase and M-1-phosphatase (M1P), which indicates that mannitol may be synthesized in these species by reduction of fructose-6-phosphate [Strandberg, 1969; Lee, 1970]. Furthermore, hexokinase activity was also measured. The levels of M1PD increased in mannitol grown cells which confirms the experiments made by Lee (1970), in which mannitol was simultaneously accumulated and dissimilated to CO_2. Indeed mannitol is a common carbohydrate reserve material in many fungi being the accumulation of free mannitol characteristic of some *Aspergillus* strains. These do not produce any mannitol from fructose, and for this reason the mannitol dehydrogenase, detected by Strandberg (1969) in *A. candidus,* is not likely to be involved in mannitol biosynthesis [Lee, 1970]. According to Hult and collaborators (1980), mannitol synthesis by reduction of F6-P is a common route in organisms belonging to the class of *Fungi Imperfecti* such as *Aspergillus*, *Botrytis*, *Penicillium*, *Piricularia*, *Trichothecium*, *Cladosporium* and *Thermomyces*. The only species of this genus lacking M1PD was the yeast *C. utilis*, which surprisingly has activity for M1P. This result also occurred for the genera *Ascomycetes*, *Gibberella*, *Ceratocystis* and *Neurospora,* that lack M1PD activity, but have activity of the subsequent reaction catalysed by M1P [Hult *et al*., 1980]. This was not the case of the genera *Phycomycetes* and *Basidiomycetes*, which lacked both M1PD and M1P [Hult *et al*., 1980]. This was in agreement with results published later by Morton and collaborators (1985) in *A. bisporus*, a basidiomycete, in which the mannitol synthesis was made from fructose catalysed by a NADPH-glycerol dehydrogenase (the alternative route for mannitol synthesis-see Figure 4.9). Production via F6-P had been earlier reported in the mould *A. alternata* [Hult and Gatenbeck, 1978].

An important finding in fungi was the report that NAD^+-linked-M1PD and M1P-phosphatase

together with hexokinase and $NADP^+$-linked-mannitol dehydrogenase, which function as a mannitol cycle, formed an important NADPH regenerating system among the fungal species belonging to the class of *Fungi Imperfecti*, which include *A. alternata* [Hult and Gatenbeck, 1978; Hult *et al*., 1980] (Figure 4.10).

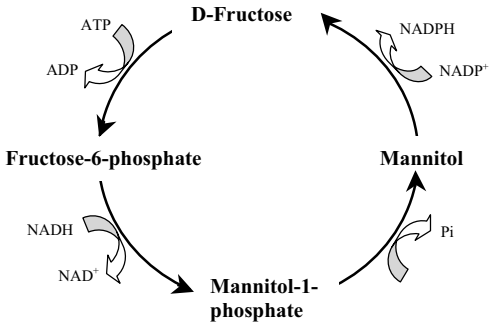


Figure 4.10. NADPH regenerating mannitol cycle in fungi. [Adapted from Hult *et al*., 1980]

This cycle is suggested to function as a transhydrogenase, an enzyme identified in mammalian cells and in cytoplasmic membrane of many bacteria, but absent from yeasts [Hoek and Rydstrom, 1988; Bruinenberg *et al*., 1983a,b]. Transhydrogenase catalyses the following reaction: NADH + $NADP^+ \Leftrightarrow NAD^+$ + NADPH, which is what happens in the cycle identified in several fungal species (Figure 4.10). The simultaneous synthesis and consumption of mannitol through that cycle leads to the generation of NAD^+ (synthesis reaction) and NADPH (consumption reaction).

4.1.6.1. *Mannitol utilization*

As described and characterized before for glycerol, mannitol may be phosphorylated through the action of a kinase or oxidated through the action of a dehydrogenase.

Dehydrogenases catalysing the oxidation of polyols may be highly specific such as glycerol dehydrogenase or less specific accepting several types of substrates such as polyols dehydrogenases. Polyol dehydrogenase with a broad substrate specificity, has been reported in the filamentous fungi: *A. niger* [Adler *et al*., 1982], *Penicillium chrysogenum* [Chiang and Knight, 1959; Adler *et al*., 1982], *S. commune* [Speth and Niederpruem, 1976], *Cephalosporium chrysogenus* [Birken and Pisano, 1976], and *Melampsora lini* [Clancy and Coffey, 1980]; and in the yeasts: *C. albicans* [Veiga *et al*., 1960], *C. utilis* [Scher and Horecker, 1966], *Pichia quercuum* [Suzuki and Onishi, 1975], and *P. stipitis* [Persson *et al*., 1994]. The enzyme from these fungi exhibits broad specificity, for example, it can use as substrate the following aldoses: D-xylose, D,L-glyceraldehyde, D-erythrose, D-ribose, L-arabinose and D-galactose. The same broad specificity has been demonstrated in *C. utilis* for polyols such as xylitol, galactitol, L-arabitol, glucitol, ribitol, erythritol and glycerol. In all cases studied thus far, the enzymes possessing dehydrogenase activity for the various polyols also retained the capacity for reduction of ketoses, with the exception of the polyol dehydrogenases purified and partially characterized in *C. chrysogenus* [Birken and Pisano, 1976] and in *M. lini* [Clancy and Coffey, 1980]. These were able to use NAD^+ or NADP as cofactors, all the other were $NADP^+$-dependent. In the case of *P. stipitis* two different polyol dehydrogenases were reported

[Persson *et al*., 1994]. One of the polyol dehydrogenases is a NAD^+-dependent specific for D-arabinitol and is a member of the short-chain dehydrogenase family, while the other belongs to the medium chain alcohol dehydrogenase family [Persson *et al*., 1994]. Both enzymes have xylitol and D-glucitol (sorbitol) as common substrates, which suggest the presence of two different families with overlapping activities [Jörnvall *et al*., 1981]. It should be noted that the

Metabolism of mannitol and other hexitols like D-glucitol (sorbitol) and D-galactitol involves specific dehydrogenases (Figure 4.9). Mannitol dehydrogenases have been studied in several organisms and two forms were described, one NAD^+-dependent and other $NADP^+$-dependent. Mannitol dehydrogenase oxidizes predominantly D-mannitol and NADPH, and reduces D-Fructose and $NADP^+$, but some of the organisms oxidizes at low rate also D-glucitol and may use also NAD^+ and NADH as cofactors. Apparently the enzyme activity is dependent on the carbon source used for growth. For instance, arabinitol dehydrogenase of *P. stipitis* showed to have significant activity for mannitol, but only in arabinitol grown cells [Hallborn *et al*., 1995], meaning that metabolism of polyols, namely polyol dehydrogenases versatility, is by far more complex than expected. Mannitol dehydrogenase NAD^+-dependent was reported in *Absidia glauca* [Ueng *et al*., 1976; Ueng and McGuinness, 1977], *A. oryzae* [Horikoshi *et al*., 1965], *Chaetomium globosum* [Adomako *et al*., 1972], *Gibberela zeae* [Trail and Xu, 2002], and *S. commune* [Niederpruem *et al*., 1965]. This enzyme is able to oxide not only D-mannitol but also D-arabitol and D-glucitol. $NADP^+$-dependent mannitol dehydrogenase was studied in *A. bisporus* [Ruffner *et al*., 1978; Morton *et al*., 1985], *Agaricus campestris* [Edmondowicz and Wriston, 1963], *A. candidus* [Strandberg, 1969], *A. oryzae* [Horikoshi *et al*., 1965], *A. parasiticus* [Niehaus and Diltz, 1982], *C. chrysogenum* [Birken and Pisano, 1976], *M. lini* [Clancey and Coffey, 1980] and *P. chrysogenum* [Boutelje *et al*., 1983].

A hexitol dehydrogenase with a broad specificity has been shown to exist in *C. utilis* [Barnett, 1968]. However, the lack in *S. cerevisiae* cells of a common dehydrogenase for mannitol and sorbitol was reported by Quain and Boulton (1987). Nevertheless, the same authors have shown that several industrial strains of *S. cerevisiae* grow well with mannitol as carbon source, and were also able to grow in sorbitol. Aerobic respiration is absolutely essential for mannitol metabolism, and in the presence of oxygen, a NAD^+-dependent mannitol dehydrogenase, highly specific for mannitol, was detected in *S. cerevisiae* [Quain and Bolton, 1987; Perfect *et al*., 1996]. Yeast unable to assimilate mannitol apparently has an impaired ability to transport this sugar alcohol. Mannitol dehydrogenase was also identified in the opportunistic pathogen *C. neoformans* [Perfect *et al*., 1996]. This yeast produces and excretes mannitol into the medium being mannitol detected in AIDS patients suffering of cryptococal meningitis [Megson *et al*., 1996]. Thus, a virulence factor has been attributed to mannitol metabolism in this yeast, although the exact nature of the role of mannitol in the development of infection is far from being understood. *C. neoformans* exhibited a cytoplasmic polyol dehydrogenase, induced by growth in mannitol, highly specific for mannitol accepting NAD^+ and $NADP^+$ as cofactors. It also reduced fructose using NADH and NADPH as cofactors. The ability of mannitol dehydrogenase to use either cofactor in *C. neoformans* suggests the presence of two isoenzymes in this yeast. Support for this suggestion comes from the inability to isolate *C. neoformans* mutants which cannot utilize mannitol. NAD^+-dependent activity has generally been associated with a catabolitic role, while $NADP^+$-dependent processes are generally considered biosynthetic. For example, *A. glauca* and bacteria utilize mannitol or fructose as their main carbon sources through the NAD^+-dependent enzyme via the mannitol cycle [Veng *et al*., 1976]. On the other hand, *A. parasiticus* and other imperfecti fungi utilize glucose to produce mannitol via the mannitol cycle with a $NADP^+$-dependent enzyme [Niehaus and Dilts, 1982]. More recently a novel mannitol dehydrogenase from a newly isolated strain of *C. magnoliae*

HH-01 was purified and characterized [Lee *et al*., 2003a]. *C. magnoliae* HH-01is the yeast strain that is currently used for the industrial production of mannitol, and has the highest mannitol production ever reported [Lee *et al*., 2003b]. Mannitol biosynthesis in *C. magnoliae* is catalysed by a NAD(P)H-dependent mannitol dehydrogenase (MDH). This enzyme catalyses both fructose reduction and mannitol oxidation, but has high substrate specificity for fructose, which may explain the high mannitol production observed in this strain [Lee et al., 2003a; Baek *et al*., 2003]. The amino acid sequence of *C. magnoliae* MDH showed a significant homology with a class of short-chain dehydrogenase reductases (SDR) from various sources, indicating that the *C. magnoliae* MDH is a NAD(P)H-dependent tetrameric SDR [Lee *et al*., 2003a]. Although mannitol dehydrogenases have been purified and characterized from several other sources, *C. magnoliae* MDH is distinguished from the other MDHs by its high substrate specificity and catalytic efficiency for fructose only, which makes *C. magnoliae* MDH the ideal choice for genetic improvement of mannitol enzymatic synthesis and salt-tolerant plants [Lee *et al*., 2003a].

The phosphorylative mannitol utilization pathway, which involves the action of a mannitol kinase followed by an oxidative step catalysed by a mannitol-1-phosphate dehydrogenase is poorly documented in fungi. Although, in bacteria the pathway for oxidation of mannitol, in which mannitol is first catabolyzed to mannitol-1-phosphate and then to fructose-6-phosphate, is operational and has been described in *E. coli* [Wolff and Kaplan, 1956], *Bacillus subtilis* [Horwitz and Kaplan, 1964], *Aerobacter aerogenes* [Luedemann, 1971], in two *Micromonospora* isolates and several representative species of other actinomycetes [Mehta *et al*., 1977]. This is not necessarily the case of fungi, in which cases there are not explicit references of the existence of mannitol kinase activity. Nevertheless, in mannitol grown cells of *A. candidus* [Strandberg, 1969], M1PD and MD activity are increased and decreased respectively, suggesting that the phosphorylative pathway is present and thus that mannitol kinase should be present.

4.1.7. *Futile cycles in fungi*

Several functions have been attributed to polyols formation but the exact purpose of many metabolism by-products and its synthesis/degradation regulation still not fully understood. These compounds with no obvious role in the internal *economy* of an organism may have a protecting physiological role or may be part of an overflow mechanism contributing to energy spilling during growth. What seems to be sure is that by-products are the result of metabolic adjustments, which are known to happen namely under stress adaptation. For instance, *D. hansenii* grown under glucose-limiting conditions and salinity changes from carbon limitation to energy limitation as the growth rate increases [Burke and Jennings, 1990]. The relationship between yield of biomass and growth rate is such that it is clear that carbon is being channelled away from anabolic to catabolic reactions. Under such conditions, energy generation can still occur at a high rate even though cell synthesis is reduced. The essential point, well known from studies on prokaryotes, is that there is uncoupling of growth and energy production.

Four metabolic cycles have been suggested to have the role of energy dissipation in fungi, namely (i) a trehalose cycle in heat shocked cells of *S. cerevisiae* [Hottiger *et al*., 1987], (ii) a mannitol cycle in many fungi [Jennings and Burke, 1990a], (iii) a glycerol cycle in *D. hansenii* growing under saline conditions [Jennings and Burke, 1990b; Adler *et al*., 1985], and (iv) a glutamine cycle in *N. crassa* under carbon limitation [Mora, 1990]. It is arguable whether these cycles are operating in a futile mode, since

they actually lead to a net production of the mentioned compounds. One possibility is that they function to fine-tuning these molecules intracellular availability, even more so if these can act as signals for signaling pathways.

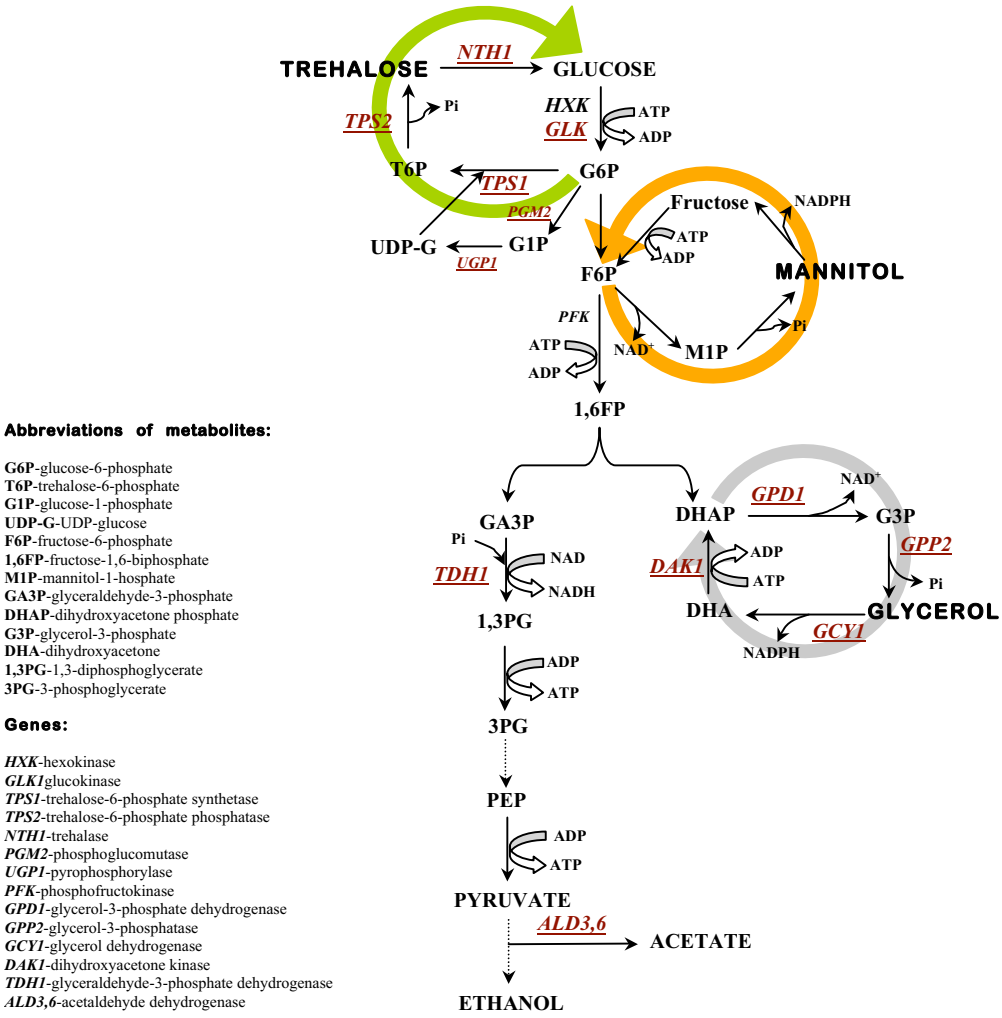
Trehalose synthesis in yeasts occurs as a side reaction at the begin of glycolysis at glucose-6-phosphate level. Yeasts possess a trehalose synthase complex which comprises both enzymatic activities required to synthesize trehalose. This complex is composed of three subunits: *TPS1* encodes the smallest, which is probably the catalytic subunit for the synthase activity which converts glucose-6-phosphate (G6P) into trehalose-6-phosphate in the presence of UDP-glucose. The *TPS2* gene encodes the phosphatases subunit and catalizes the conversion of trehalose-6-phosphate into trehalose (Figure 4.11). Both *TSL1* and *TPS3* redundantly encode the largest subunit of the enzyme [Thevelein and Hohmann, 1995]. UDP-glucose is produced from G6P in a parallel reaction involving two steps, being the first catalysed by a phosphoglucomutase (encoded by *PGM1* and *PGM2* genes) followed by the action of a UDP-glucose pyrophosphorylase (encoded by *UGP1* and *YHL012* genes). On the other hand, trehalose can be used as carbon source through the action of a trehalase encoded by *NTH1* gene (Chapter 2). In the sense that trehalose can be produced or used as a carbon source feeding glycolysis, this cycle is rather similar to the cases of glycerol and mannitol described above (Figure 4.11).

Actively proliferating cells that experience a hyperosmotic shock will face growth arrest depending on the shock severity. One of the consequences of this rapid decrease in molecular synthesis is a dramatic decrease in the ATP demand from biosynthesis. It has recently been proposed that under conditions of growth arrest, with low ATP demand, cells face the threat of substrate-accelerated death [Teusink *et al*., 1998]. The reason for this is the turbo design of the upper part of glycolysis, where two molecules of ATP are invested yielding a total outcome of four ATP molecules per glucose utilized, in the lower part. This increase net production of ATP will stimulate the flux in the upper part of glycolysis, and thus leads to an imbalance between the upper and lower parts. The theoretical result in this non-regulated case is a dramatic and steady accumulation of the intermediates hexose phosphate and fructose-1,6-bisphosphate, probably leading ultimately to phosphate depletion and cell death. Trehalose and glycerol production during growth has been related with the cells necessity to restore the inorganic phosphate levels needed for glycolysis prosecution [Luyten *et al.*, 1995]. To overcome the ATP overproduction problem, glycolysis should stop, but cells need to osmoregulate by glycerol production and thus require a flux in glycolysis. Thus being Blomberg proposed a metabolic model in which glycerol and trehalose cycles functioning as glycolytic safety valves or *energy spillage* mechanisms, by spending the ATP in excess and thus promoting a *normal* flux through glycolysis, during cellular stress adaptation. There is experimental evidence for trehalose and glycerol turnover during cellular adaptation to different environmental stresses [Parrou *et al*., 1997; Godon *et al.*, 1998; Hottiger *et al*., 1987]. In addition, expression of trehalase (*NTH1*) and dihydroxyacetone kinase (*DAK1*) are induced by a number of other stresses besides osmotic dehydration [Godon *et al*., 1998; Zähringer *et al*., 1997; Jelinsky and Samson, 1999] (Figure 4.11).

With regard to salt stress, recent publications using 2D-PAGE and microarrays in *S. cerevisiae* cells (Chapter 1) showed that mRNA expression levels of enzymes belonging to trehalose and glycerol futile cycles represented in Figure 4.11, were increased upon osmotic shock [Norbeck *et al*., 1996; Rep *et al*., 2000, Causton *et al*., 2001; Posas *et al*., 2000; Yale *et al.*, 2001]. Although these results cannot be interpreted as enzymatic activity, since there may exist several regulatory mechanisms after transcription, they constitute good evidences for the existence of operative futile cycles as proposed before. For instance the gene *GCY1* encoding a putative glycerol dehydrogenase in *S. cerevisiae*

increases in mRNA expression under stress but all the attempts made to measure the enzyme activity were without success [Norbeck and Blomberg, 1997]. In Figure 4.11 is also represented a mannitol production/consumption cycle. Up to date no such cycle has been suggested in *S. cerevisiae*, since this yeast does not produce this compound. Though, as said in Chapter 2 many yeasts accumulate mannitol when growing in carbon sources such as glucose, glycerol and ethanol. Indeed, mannitol cycle has been suggested as a futile cycle in fungi [Jennings and Burke, 1990].

Figure 4.11. Putative futile cycles in fungi. Salt induced genes are underlined. [Adapted from Blomberg, 2000]



It is interesting to note that trehalose cycle displays one main difference from glycerol and mannitol cycles, in that these two last also involve redox-coupled reactions. For instance mannitol cycle was proposed in *Imperfecti Fungi* as an important NADPH regenerating system [Hult and Gatenbeck, 1980]. Most important is the fact that if the enzymes represented in Figure 4.11 use the

cofactors indicated glycerol and mannitol may be functioning to substitute a transhydrogenase function. Mannitol and glycerol cycles may be fulfilling simultaneously different roles such as compatible solutes production, fine tuning of the osmolyte intracellular concentration, ATP futile cycles and redox balancing [Adler *et al*., 1989; Jennings and Burke, 1990; Parrou *et al*., 1997; Teusink, 1999; Blomberg, 2000; Costenoble *et al*., 2000]. Theoretical models and a better understanding of metabolic networks in operation in yeasts will be essential for testing the model proposed above.

4.1.8. *Redox balance in the metabolism of yeasts*

In order to proliferate cells must divide and new building metabolic blocks must be produced, such as nucleic acids, lipids, and so on. The necessity of redox balance arises from the formation of new biomass, since biomass formation is accompanied by a net consumption of NADPH and a net production of NADH [Albers *et al*., 1996; van Dijken and Scheffers, 1986]. Thus, maintenance of intracellular redox balance by controlling the cytosolic NADH/NAD^+ and NADPH/$NADP^+$ ratios is crucially important for sustained oxidation of cellular substrates during growth. In other words, NADH and NADPH generated in the cytosol must be re-oxidized to NAD^+ and $NADP^+$. Reducing equivalents transferred through these cofactors will allow, depending on the type of metabolism, the generation of energy in the form of ATP during glycolysis or in the mitochondrial electron chain transporter. The exact magnitude of NADH generated and NADPH consumed as a consequence of biomass formation is dependent on the biomass composition and the extent to which exogenous lipids contribute to the overall lipid content of the yeast cells.

In the metabolism of sugars by yeasts NAD(H) and NAD(P)H play separate and distinct roles. NADH is regarded as being mainly involved in catabolic processes whereas NADPH is associated frequently with anabolic processes [van Dijken and Scheffers, 1986; Bakker *et al*., 2001]. However this is not always the case. In fact, the distinction between assimilatory and dissimilatory reactions in heterotrophic organisms is to some extend artificial. For example, glycolysis plays as essential role in sugar dissimilation, but also generates building blocks for biosynthesis. Furthermore, although most biosynthetic reactions use NADPH as a reductant, some NADH-linked reactions occur in the conversion of central metabolites (pyruvate, oxaloacetate, acetyl-CoA) to cellular monomers such as amino acid biosynthesis. Besides their physical compartmentation in cytosol and mitochondria that occurs as a consequence of the impermeability of the mitochondrial inner membrane for NADH, NADPH, NAD^+ and $NADP^+$, the two coenzyme systems are separated by chemical compartmentation due to the absence of transhydrogenase activity. This aspect has considerable consequences for the redox balances of both coenzymes systems and hence for metabolism in yeasts, having significant influence on product formation during growth. The enzyme pyridine nucleotide transhydrogenase, which catalyses transfer of reducing equivalents between the two nucleotide systems, according to the reaction NADH + $NADP^+$ $\Leftrightarrow$ NAD^+ + NADPH, exhists in several organisms like mammalian cells and in the cytoplasmic membrane of bacteria such as *E. coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Azotobacter vinelandii*, but not in yeasts [Hoek and Rydstrom, 1988; Bruinenberg *et al*, 1983a,b]. In the absence of such systems, yeasts must possess specific mechanisms for the re-oxidation of reduced cofactors both in cytosol and mitochondria and also connecting these two compartments. Occurrence of separate mitochondrial and cytosolic NADH/NAD^+ and NADPH/$NADP^+$ redox couples is not only relevant during aerobic, respiratory growth, but also during anaerobic, fermentative growth.

NADPH production and consumption in yeasts

NADPH production and consumption is strongly dependent on the carbon and nitrogen source available for growth. Lipid biosynthesis is the major NADPH-consuming process and occurs in the cytoplasm [Botham and Ratledge, 1979]. Except for some mitochondrial reduction steps in the biosynthesis of arginine, valine, leucine and isoleucine, the NADPH-consuming reactions of amino acid synthesis are also located in the cytoplasm [Bruinenberg *et al*., 1983a,b]. When nitrate is used as nitrogen source the enzymes responsible for its reduction occurs in the cytoplasm and uses NADPH or NADH depending on the strain used. Since most of NADPH consuming processes occur in the cytoplasm, the localization of NADPH-producing processes in the yeast cell is important. The oxidative part of the pentose phosphate pathway, a $NADP^+$-linked isocitrate dehydrogenase and the $NADP^+$-dependent acetaldehyde dehydrogenase have been suggested to be the major routes of NADPH formation in glucose or ethanol-grown cells of *S. cerevisiae* and *C. utilis* [Gancedo and Lagunas, 1973; Bruinenberg *et al*., 1983a,b; Minard *et al*., 1998]. In certain moulds a mannitol cycle has been postulated as a source of cytoplasmic NADPH [Hult and Gatenbeck, 1980]. This transhydrogenating system, which requires the net input of one ATP per NADPH, has so far not been detected in yeasts. One interesting finding is that several crucial antioxidant cellular mechanisms are dependent on NADPH [Minard and MacAlister-Henn, 2001]. In the case where these reactions generate more NADPH than the one used in assimilatory reactions, redox balancing requires an additional NADPH-oxidizing process. Mitochondrial NADPH dehydrogenases, which couple oxidation of cytosolic NADPH to the mitochondrial respiratory chain, are common in plants [Møller and Lin, 1986], and have been reported in fungi [Melo *et al*., 2001], in the yeasts *C. utilis* [Bruinenberg *et al*., 1985, van Urk *et al*., 1989a], *S. carlsbergensis* [Schuurmans Stekhoven, 1966], and *K. lactis* [Overkamp *et al*., 2002b], but is absent from *S. cerevisiae* [de Vries and Marres, 1987; van Urk *et al*., 1989a; Small and McAlister-Henn, 1998]. This is in agreement with the hypothesis that the *S. cerevisiae pgi1*Δ mutated in a phosphoglucose isomerase, cannot grow on glucose as sole carbon source, because it cannot oxidize the surplus of NADPH. The same type of mutation in *K. lactis* allowed growth in glucose, and this was suggested to be due to the ability of this yeast to oxidize NADPH through the mitochondria [Small and McAlister-Henn, 1998].

NADH production and consumption in yeasts

Based on the biomass composition of *S. cerevisiae*, it has been estimated that some 60-80% of the NADH generated in biosynthetic reactions originates from the synthesis of amino acids [Bruinenberg *et al*, 1983a,b; Albers *et al*., 1998]. Although the localization of the enzymes is not known in all cases, rough calculations show that 30-50% of the NADH produced by amino acid synthesis is generated in the mitochondria [Albers *et al*., 1998]. Nissen and collaborators (1997) performed a quantitative flux analysis for anaerobically growing *S. cerevisiae* and showed that a large part of the mitochondrial NADH is derived from synthesis of 2-oxoglutarate, which is a precursor of glutamate. While the synthesis of other amino acids from glutamate or other precursors contributes to a lesser extent to mitochondrial NADH production, the DNA and RNA synthesis seems to involve only cytosolic NADH and NADPH [Nissen *et al*., 1997]. In addition to the formation of biomass, also the excretion of oxidized low-molecular metabolites (*e.g*. pyruvate, acetaldehyde or acetate) during growth on glucose may lead to a net production of NADH [van Dijkens and Scheffers, 1986; Nissen *et al*., 1997].

Due to the impermeability of mitochondria inner membrane to pyridine nucleotide

coenzymes [van Jagow and Klingenberg, 1970], the re-oxidation of reduced coenzymes must occur in the compartment where they are generated. In contrast to NADPH turnover, which occurs predominantly in the cytosol [Bruinenberg *et al.*, 1983a,b; Albers *et al.*, 1998], NADH-turnover occurs at high rates in the cytosol as well as in the mitochondrial matrix. Under aerobic conditions, NADH is generated in the cytosol by glycolysis as well as in the mitochondrial matrix by enzymes of the tricarboxylic acid cycle. Both pools of NADH can be oxidized by the mitochondrial respiratory chain with oxygen as the terminal electron acceptor [de Vries and Marres, 1987; Luttik *et al.*, 1998; Overkamp *et al.*, 2000; Bakker *et al.*, 2001; Påhlman *et al.*, 2001b]. Since under anaerobic conditions the re-oxidation of NADH is made through glycerol production [van Dijken and Scheffers, 1986], and the enzymes involved in glycerol synthesis are exclusively cytosolic, anaerobic re-oxidation of intramitochondrial NADH requires a shuttle mechanism that exports redox equivalents to the cytosol. The relative rates of NADH turnover in the cytosol and the mitochondrial matrix strongly depend on the carbon source and some mechanisms exist that couple the oxidation of NADH in these two compartments to the respiratory chain. There are at least five mechanisms of NADH re-oxidation in *S. cerevisiae*: (1) alcoholic fermentation, (2) glycerol production, (3) respiration of cytosolic NADH via external mitochondrial NADH dehydrogenase, (4) respiration of cytosolic NADH via the glycerol-3-phosphate shuttle, and (5) oxidation of intramitochondrial NADH via a mitochondrial internal NADH dehydrogenase [Bakker *et al.*, 2001]. Furthermore, *in vivo* evidence indicates that NADH redox equivalents can be shuttled across the mitochondrial inner membrane by an ethanol-acetaldehyde shuttle [Bakker *et al.*, 2001]. Several other redox-shuttle mechanisms might occur in *S. cerevisiae*, including a malate-oxaloacetate shuttle, a malate-aspartate shuttle and a malate-pyruvate shuttle [Bakker *et al.*, 2001]. Although key enzymes and transporters for these shuttles are present, there is yet no consistent evidence for both their *in vivo* activity and their role in yeast metabolism.

Yeast mitochondria oxidize cytosolic NADH preferentially and directly by the action of an external NADH dehydrogenase localized in the inner mitochondrial membrane and has its catalytic site faced to the mitochondrial intermembrane space [de Vries and Marres, 1987; Påhlman *et al.*, 2001b]. In *S. cerevisiae* these enzymes are encoded by two typical aerobic genes *NDE1* and *NDE2* [Small and McAlister-Henn, 1998; Luttik *et al.*, 1998]. *NDE1* is physiologically more important than *NDE2*, and deletion of both genes is somehow substituted by other type of mechanisms such as the glycerol-3-phosphate shuttle [Larsson *et al.*, 1998]. Internal NADH dehydrogenase as its catalytic site faced to the mitochondrial matrix, is subjected to glucose repression, specific for NADH and is encoded in *S. cerevisiae* by *NDI1* gene [Marres *et al.*, 1991]. Disruption of the gene *NDI1*, the only internal NADH dehydrogenase found in S. cerevisiae, have no effect on growth with fermenTable carbon sources or ethanol, but eliminate growth on carbon sources that generate NADH in the mitochondrial matrix such as lactate, pyruvate and acetate [Marres *et al.*, 1991].

Physical transport of NAD^+ and NADH across the mitochondrial inner membrane exist, but proceeds at extreme low rate and furthermore is insignificant in the redox cycling [Averet *et al.*, 2002]. Thus, many eukaryotic cells harbor redox shuttles, which transfer the redox equivalents across the mitochondrial membrane. The glycerol-3-phosphate shuttle is an indirect mechanism to oxidize cytosolic NADH and transfer the electrons to the respiratory chain. This shuttle consists, in *S. cerevisiae,* of two components: cytosolic NAD^+-linked glycerol-3-phosphate dehydrogenase, encoded by *GPD1* and *GPD2* genes (see section 4.1.6.1) and mitochondrial FAD-linked glycerol-3-phosphate dehydrogenase, encoded by *GUT2* and localized in the inner mitochondrial membrane (Figure 4.12-represented in green). Since *GUT2* is repressed by glucose, deletion of this gene only produced substantial amounts of glycerol in ethanol-grown cells, meaning that the G3P shuttle is important in this type of

carbon source, and when interrupted, cells are forced to re-oxidize their cytoplasmic NADH through the glycerol synthesis. *GUT2* in aerobic conditions with glucose as carbon source is under control of NADH dehydrogenase [Påhlman *et al*., 2002], and repressed by glucose, thus G3P shuttle is probably more useful under anaerobic conditions with non repressible carbon sources or as an alternative re-oxidation mechanism.

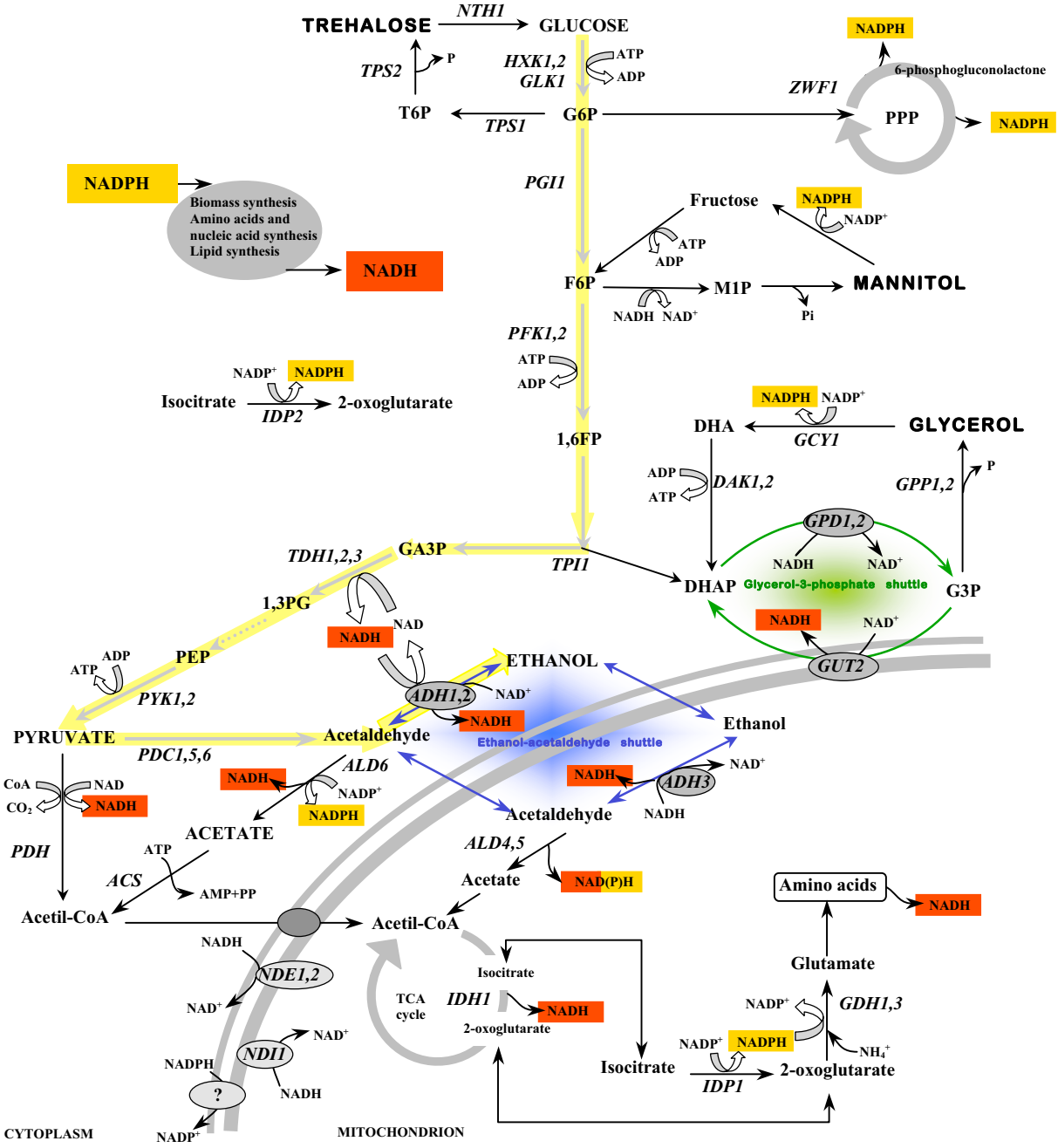
As said before ethanol-acetaldehyde shuttle can export redox equivalents from the mitochondrial matrix to the cytosol and thus allow respiratory growth of a mutant in the gene coding for NADH dehydrogenase (*NDI1*). Ethanol and acetaldehyde diffuse freely across biological membrane and thus the shuttle is symmetric (Figure 4.12-represented in blue). In principle ethanol-acetaldehyde shuttle is reversible being dependent on the NADH/NAD^+ ratios in the cytoplasm and in the mitochondrial matrix. This shuttle does not seem to be operational in respiring wild-type cultures, however may play a key role in the re-oxidation of mitochondrial NADH in anaerobic cultures [Bakker *et al*., 2000], since deletion of the alcohol dehydrogenase *ADH3* implied a reduction of 30% in the specific growth rate of anaerobic glucose-grown cells.

In summary:

The major sources of NADPH (Figure 4.12) are the Pentose Phosphate Pathway (PPP), the $NADP^+$-dependent isocitrate dehydrogenase, both cytoplasmic (*IDP2*) and mitochondrial (*IDP1*) and the acetaldehyde dehydrogenase cytoplasmic $NADP^+$-dependent (*ALD6*). Blocking the pentose phosphate pathway turns the production of acetate through the *ALD6* product gene the most important source of NADPH in the cell. NADPH may also be generated through the putative futile cycle of glycerol and mannitol resulting from the action of a glycerol (*GCY1*) and a mannitol dehydrogenase (Figure 4.12). There are not clear evidences that *S. cerevisiae* possess a futile mannitol cycle, since some strains do not use mannitol, although some yeasts produces and uses mannitol, and has a possible NADPH supplier and as an hypothetic transhydrogenating system (suggested in fungi) it is also represented (Figure 4.12). NADPH is mainly used in biomass production, amino acids, nucleic acids and in lipid synthesis and must be generated and re-oxidized both in the cytoplasm and in the mitochondrial matrix (Figure 4.12). The conversion of 2-oxoglutarate into glutamate by glutamate dehydrogenase is also one of the major NADPH consuming reaction (Figure 4.12). In mitochondria the conversion of isocitrate into 2-oxoglutarate by the product of the gene *IDH1* (isocitrate dehydrogenase NAD^+-dependent) is the major supplier of NADH. NADH is re-oxidized essentially through the mitochondrial NADH dehydrogenases (*NDE1,2*), in aerobic conditions, whereas in anaerobic conditions NADH surplus is re-oxidized through the glycerol synthesis (Figure 4.12). Mitochondrial NADH re-oxidation is made by an internal NADH dehydrogenase (*NDI1*).

Figure 4.12. Mechanisms at the interface between the NADPH and NADH-yielding reactions in assimilatory and dissimilatory sugar metabolism and the mitochondria respiratory chain in the yeast *S. cerevisiae*. Ethanol-acetaldehyde shuttle is represented in blue. Glycerol-3-phosphate shuttle is represented in green. Glycolysis is represented in yellow. Yellow and red boxes highlights reactions where NADPH and NADH are produced respectively. **Abbreviations of metabolites**: **G6P**-glucose-6-phosphate; **T6P**-trehalose-6-phosphate; **F6P**-fructose-6-phosphate**; 1,6FP**-fructose-1,6-biphosphate; **M1P**-mannitol-1-hosphate; **GA3P**-glyceraldehyde-3-phosphate; **DHAP**-dihydroxyacetone phosphate; **G3P**-glycerol-3-phosphate; **DHA**-dihydroxyacetone**; 1,3PG**-1,3-diphosphoglycerate; **PEP**-phosphoenolpyruvate; **Genes:** ***HXK***-hexokinase; ***GLK1***glucokinase; ***TPS1***-trehalose-6-phosphate synthetase; ***TPS2***-trehalose-6-phosphate phosphatases; ***NTH1***-trehalase; ***PGI1***-phosphoglucose isomerase; ***ZWF1***-glucose-6-phosphate dehydrogenase; ***PFK***-phosphofructokinase; ***TPI1***-triosephosphate isomerase; ***IDP2***- cytoplasmic $NADP^+$-dependent isocitrate dehydrogenase; ***GPD***-glycerol-3-phosphate dehydrogenase; ***GPP***-glycerol-3-phosphatase; ***GCY1***-glycerol

dehydrogenase; ***DAK***-dihydroxyacetone kinase; ***GUT2***-mitochondrial FAD^+-dependent glycerol-3-phosphate dehydrogenase; ***TDH***-glyceraldehyde-3-phosphate dehydrogenase; ***PYK***-pyruvate kinase; ***PDC***-pyruvate decarboxylase; ***PDH***-pyruvate dehydrogenase complex; ***ACS***-Acetil-CoA synthetase; ***ALD6***- cytoplasmic $NADP^+$-dependent acetaldehyde dehydrogenase; ***ADH1,2***-cytoplasmic NAD^+-dependent alcohol dehydrogenases; ***ADH3***-mitochondrial NAD^+-dependent alcohol dehydrogenase; ***ADH4,5***-mitochondrial alcohol dehydrogenases; ***IDH1***-mitochondrial NAD^+-dependent isocitrate dehydrogenase; ***IDP1***-mitochondrial $NADP^+$-dependent isocitrate dehydrogenase; ***GDH***- $NADP^+$-dependent glutamate dehydrogenase; ***NDI1***-internal NADH dehydrogenase; ***NDE***-external NADH dehydrogenases.



Redox metabolism cannot be viewed as an isolated process: its regulation is closely connected to central and peripheral reactions in carbon and nitrogen metabolism. In spite of the literature on the subject, there is still missing knowledge on this matter, in particular because each organism establishes a different equilibrium according to the growth conditions through parallel pathways for re-oxidation of reduced coenzymes.

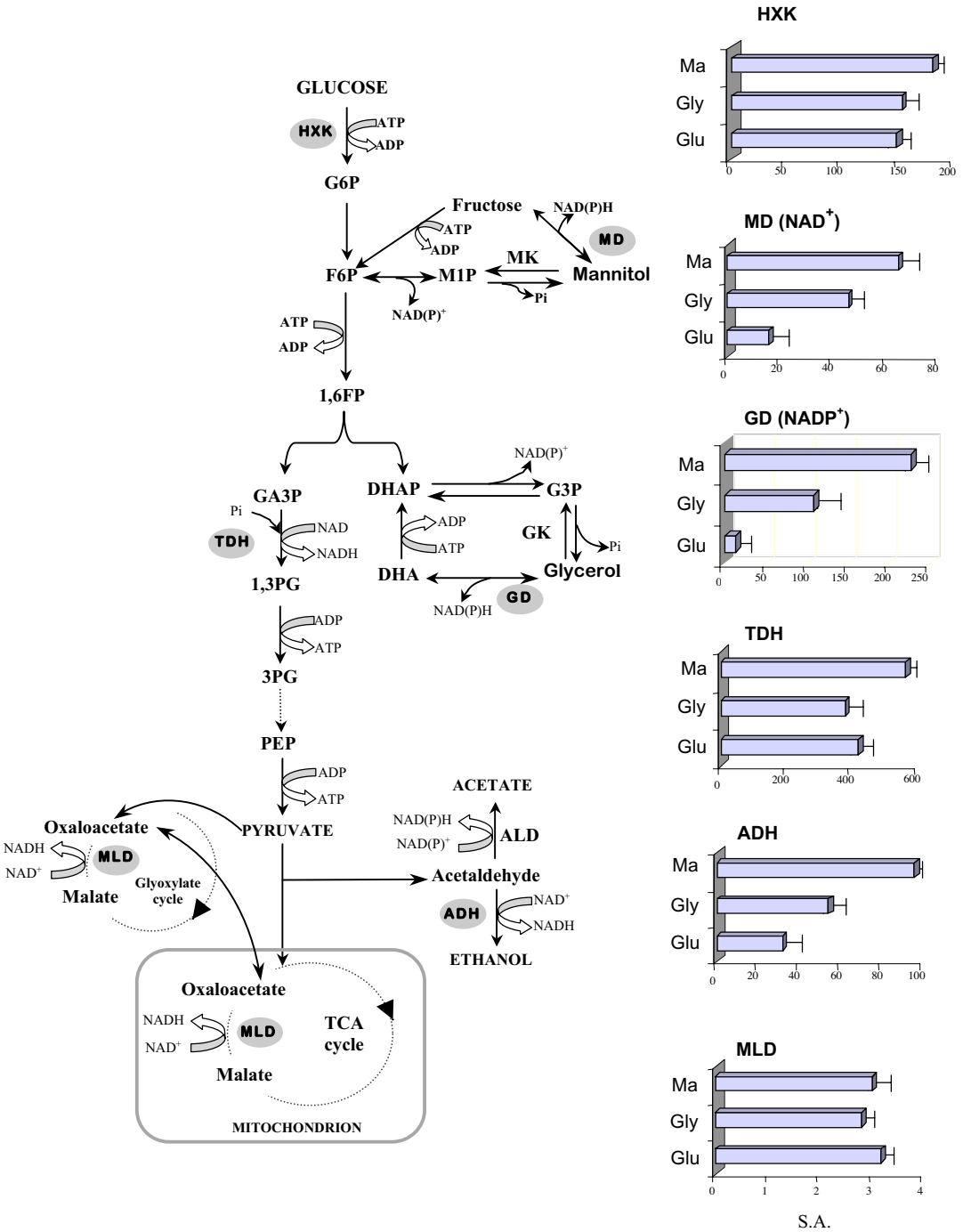
4.2. Results and discussion

We have shown in Chapter 2 that *C. halophila* grows on glucose, glycerol, mannitol and ethanol as carbon and energy sources. This yeast evidenced the use of a respiro-fermentative metabolism, in which glycerol, mannitol, ethanol and acetate were produced as by-products. In addition, when submitted to heavy salt stress, *C. halophila* confirmed the use of a respiro-fermentative metabolism, being both the oxygen consumption and carbon dioxide production enhanced, although to different extent. The increase in CO_2 production was accompanied by an increase in ethanol production. To corroborate these findings we decided to measure the specific activities of enzymes that we considered relevant as first approach towards the understanding of *C. halophila* metabolism under salt stress conditions.

4.2.1. *Enzyme activities of main metabolic pathways*

Since *C. halophila* respires and ferments at the same time, it will be expected to measure specific activity of enzymes assigned with the production of ethanol and with Krebs cycle. Thus we selected the following enzymes as the first approach to metabolism characterization: hexokinase – as the first enzyme in the use of glucose and a key enzyme in regulation of glycolytic flux; glyceraldehyde-3-phosphate – a highly expressed enzyme in *S. cerevisiae* and an important catalytic step during glycolylis due to its localization subsequent to the ramification point, in which glycerol may be formed; cytoplasmic ethanol dehydrogenase – an obligatory enzyme in fermentation leading to ethanol production and malate dehydrogenase – as an enzyme belonging to the Krebs cycle. In addition we searched also for glycerol and mannitol kinase and dehydrogenase activities as potential enzymes in the utilization of both these compounds as carbon sources. All these enzymes were assayed in glucose, glycerol and mannitol-grown cells being the results presented in Figure 4.13. As expected from the oxygen consumption and carbon dioxide production rates measured before (Chapter 2), *C. halophila* is using a respiro-fermentative metabolism with either carbon source tested, since both alcohol dehydrogenase and malate dehydrogenase specific activities were determined. We cannot assume that the malate dehydrogenase activity determined corresponds to a mitochondrial enzyme form. As presented before in section 4.1.3, *S. cerevisiae* has three malate dehydrogenases characterized, which are localized in different cellular compartments. Since we assayed the malate dehydrogenase in cell free extracts, most probably we are essentially measuring soluble cytoplasmic enzyme activity, and to a less extent the mitochondrial form. In the case of the constitutive expression of the three forms, activity measurements should give high values, corresponding to the sum of the activity of the individual forms. However, we do not know if *C. halophila* has several forms of malate dehydrogenase.

Figure 4.13. Schematic representation of glycolysis, ethanol fermentation and Krebs cycle and the specific activity of enzymes in cell free extracts of *C. halophila* cells grown in different carbon sources. Ma- mannitol grown cells; Gly- glycerol grown cells; Glu- glucose grown cells. **HXK**- hexokinase; **MD** – Mannitol dehydrogenase; **GD** – Glycerol dehydrogenase; **TDH** – Glyceraldehyde-3-phosphate dehydrogenase; **ADH**- Alcohol dehydrogenase and **MLD** – malate dehydrogenase. S.A. Specific activity in mU mg^{-1} protein (U = µmol min^{-1}), except for malate dehydrogenase (MLD) in which activity was expressed as U mg^{-1} protein.



If in one hand the probability that the specific activity of malate dehydrogenase measured corresponds to a cytoplasmic form involved in glyoxylate cycle is high, on the other hand, we have determined the rates of oxygen consumption, and thus, even if the results obtained do not correspond to a Krebs cycle enzyme, this cycle must be working at some extent. It is well known that sugar catabolism in aerobic batch systems places serious oxygen availability restrictions, and as a consequence many yeasts exhibit in these conditions a respiro-fermentative metabolism. While activity of malate dehydrogenase is similar among the carbon sources tested indirectly suggesting a constant respiratory branch, alcohol dehydrogenase differed considerably, being the highest value measured in mannitol grown cells. Indeed, most of the enzymes assayed have their values enhanced in mannitol grown cells. This may be related with the ability to reduce substantially the metabolic repression caused by easy fermentable carbon sources such as glucose.

We also attempted to measure activity of acetaldehyde dehydrogenase (ALD) without success. In *S. cerevisiae,* of the three cytoplasmic acetaldehyde dehydrogenases identified, only the one $NADP^+$-dependent is constitutively expressed, while the other is glucose repressed. It is surprising that no activity could be detected in *C. halophila* extracts, since acetate was measured intracellularly in any of the carbon sources used. Most probably this was due to some experimental procedure inadequacy.

Hexokinase, assayed with glucose as substrate, produced similar results in cells cultured in either carbon source (Figure 4.13). In addition we also tested this enzyme using fructose, and the results were identical (not shown). This means that the enzyme is constitutively produced. It is not likely that there are two isoenzymes, for glucose and fructose, since the specific activity measured with either substrate in cells cultured in either carbon source were identical. The regulation exerted by hexokinases on glucose metabolism (as in other carbon sources) is still controversial and follows a complex pattern [Gancedo, 1998; Flores *et al*., 2000]. Their metabolic localization at the beginning of glycolysis is consistent with a complex regulatory phenomena branch-point.

The presence of activity for hexokinase in mannitol and glycerol grown cells may be related to the production of intermediates such as fructose, which may be further dissimilated through the action of the hexokinase. This hypothesis fits well with the fact that *C. halophila* glycerol-grown cells produce mannitol, which can be dissimilated through the production of fructose via a glycerol dehydrogenase (Figure 4.13). In the case of mannitol grown cells, mannitol may be also utilized through the fructose pathway. This is probably the case, since hexokinase has the highest activity values in mannitol grown cells.

C. halophila showed the ability to grow with similar growth rate in media with glucose, glycerol or mannitol as carbon and energy sources. According to the revision made in the earlier sections, there are, among the yeasts, two types of enzymes that may be used to polyol dissimilation namely kinases or dehydrogenases. We began by searching for glycerol and mannitol kinase activity in cells cultured in either substrate without success. Kinase activity is not always straightforwardly detecTable. In *S. cerevisiae* there are two types of assays published to detect glycerol kinase activity, one is spectrophotometric and the other is based on radiolabeled product formation measurement [Adler *et al*., 1985]. Both types of protocols were implemented in *S. cerevisiae* glycerol grown cells used for procedure control. Results (not shown) were obtained with the spectrophotometric protocol. In *C. halophila* result was negative. This led us to try to assay the glycerol and mannitol dehydrogenases, from the alternative dissimilation pathways (Figure 4.13). Dehydrogenase activity was determined for glycerol and mannitol as substrates in the three carbon sources tested, although these polyols revealed to use different cofactors. Apparently, mannitol is dissimilated through a mannitol dehydrogenase in a

NAD^+-dependent way, while glycerol is in a $NADP^+$ dependent way (Figure 4.13). Glucose-grown cells showed the lowest values for both these enzymes. This suggests that in spite of being constitutively expressed, these enzymes are not without regulation. Finally, we have to comment on the fact that the enzymatic assays do not ultimately allow us to distinguish between the possibility of glycerol and mannitol dehydrogenase activities being performed by one single enzyme or by two distinct ones, although the co-factor specificity suggests that. This possibility can only be clarified when the corresponding genetic sequences are retrieved and mutants are available. In the meantime, we can face a last possibility of this being the activity of a polyol dehydrogenase. One such enzyme has been characterized in some yeast (Section 4.1.6.12). Nevertheless, this enzyme shows high affinity for arabitol, xylitol and sorbitol but not for mannitol and glycerol. This subject will be further approached.

In Chapter 2 we have shown that glucose and glycerol were consumed sequentially in a medium containing equal moles of each substrate. In Chapter 3 we have shown that glycerol and glucose, both were transported through constitutive transport systems. In this chapter we show that the first enzyme in these substrates metabolism is also constitutively expressed. Yet, both glycerol transport and GD activity are under some degree of down-regulation when the cells are growing on glucose, though it does not appear enough to explain the inability of the cells to consume both substrates at the same time. The same is true for glucose-mannitol mixture. Glycerol-mannitol mixture proved to correspond to simultaneous consumption of the two substrates and the transport (Chapter 3) and enzymatic activity measurements support that result.

Comparing the specific activities of glyceraldehyde 3-P dehydrogenase (TDH), alcohol dehydrogenase (ADH) and malate dehydrogenase (MLD) in cells cultivated in either carbon source it becomes evident that the degree of variation in specific activity of these three enzymes with carbon source is not identical, though they are in accordance with the respiro-fermentative behaviour of *C. halophila* (Chapter 2). While MLD stays approximately constant, TDH increases moderately in mannitol-grown cells in comparison to glycerol- and glucose-grown cells and ADH increases steeply from glucose- to glycerol- and to mannitol-grown cells. The steep increase in either glycerol or mannitol consumption enzymes compared to glucose's, observed in mannitol-grown cells, would correspond to general glycolytic flux being channeled to fermentation. Whether this is redox-coupled, due to GD and MD co-factor specificity, we can only suggest. Anyway, this remains a possibility since, in opposition to the case of glycerol consumption in *S. cerevisiae*, in *C. halophila* glycerol is, according to our results, consumed through dihydroxacetone pathway.

4.2.2. *Enzyme activities of main metabolic pathways in salt-stress grown cells*

Salt stress imposes to microorganisms cells a remodelation of their entire metabolism in order to proliferate. Cells will have only an ability to stay alive if their metabolism is able to counteract the deleterious effects caused by osmotic stress to the cell. Many studies concerning the expression of a large set of genes, have been recently published using short exposure time scales [Rep *et al*., 2000; Posas *et al*., 2000; Yale *et al*., 2001; Causton *et al*., 2001]. Several genes showed to increase their RNA expression either transiently or permanently, but the specific activity of enzymes associated with specific genes has been only determined in few cases, and thus generally, it is not known if the RNA expression levels correspond to an effective active gene product (Chapter 1). We determined the specific activity of some of the enzymes already determined before in glucose, glycerol and mannitol grown cells, this time in glucose-grown cells in the presence of increasing salt concentrations in the medium.

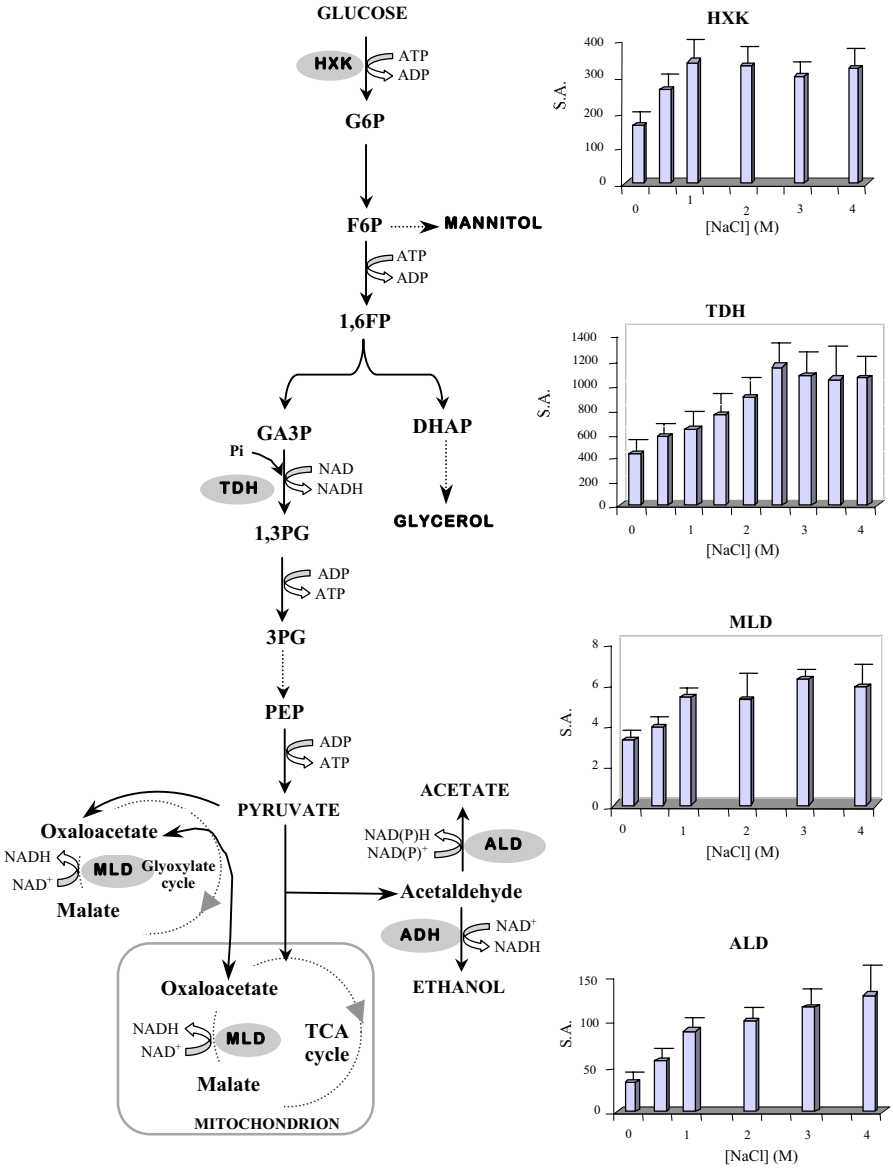
Our goal was to access under stress conditions the type of metabolism that *C. halophila* was performing, since the results in Chapter 2 showed a general increase in both respiratory and fermentative fluxes in cells cultivated under such circumstances.

Cell free extracts were obtained from salt grown cells harvested in late exponential phase. No salt has been added to any enzymatic assay. All the enzymes assayed presented activity. The specific activity followed a general pattern of increase in cells cultured under increasing stress (Figure 4.14) consistent with the increase in respiration and fermentation mentioned above. Furthermore, this increase suggests that *C. halophila* not only grows well under salt stress, but also it *improves* the metabolism under such circumstances. These results are consistent with the increase observed in μ_g on glucose with ≤2M NaCl (Chapter 2, figure 2.8A).

According to the Figure 4.14, although an increase in specific activities has been observed in the four enzymes assayed the degree of increase in activity was not identical for all the enzymes. Hexokinase (HXK) and malate dehydrogenase (MLD) activities increased progressively up to 1M NaCl and then remained constant, whereas TDH activity increased up to 2.5M NaCl and then stabilized their values. Ethanol dehydrogenase behaved slightly different since its activity increased constantly up to 4M NaCl. In Chapter 2 we showed that *C. halophila* cells have their CO_2 and ethanol production progressively increased in media containing increased salt concentrations, reaching ± 400% in 4M NaCl. These results are corroborated by the determination of ethanol dehydrogenase activity under stress, which showed also an increase of about 400%. Furthermore the progressive increase in ADH is very similar with the increase presented for CO_2 production rates, reason why it seems correct to roughly state that CO_2 produced is mainly the result of enhanced fermentation and only a minor percentage may be attributed to respiration through the Krebs cycle. *C. halophila* cells grown with increasing salt concentrations showed to produce acetic acid in a linear relation with salt in the medium (Chapter 2). Nevertheless, we were not able to measure any specific activity of ALD-acetaldehyde dehydrogenase in salt-grown cells. We have discussed earlier that malate dehydrogenase activity measured is probably due to a cytoplasmic and not a mitochondrial enzyme however, in the presence of salt, MDL specific activity increased up to 180%, which is very similar to the increase observed for oxygen consumption rates under stress (about 150%). A possibility is that we are simultaneously measuring both isoforms which somehow may justify the higher values of specific activity found for MLD when compared with the other enzymes as already mentioned above. We cannot forget that we are assuming that *C. halophila* may have more then one MLD isoforms localized in different cellular compartments.

Results concerning the specific activity of TDH obtained in salt-grown cells are very interesting since they increased up to a concentration of salt in the medium which was assigned before in Chapter 2 as an important inflexion point in physiological growth of *C. halophila* cells. Above 2.5M NaCl growth rate of *C. halophila* cells started to decrease exponentially, although the biomass values remained almost constant. This behavior accompanies the variation of μ_g over the same salinity range. Above 2.5M NaCl growth rate decreases and TDH activity arrests. The reason underlying these results is not straightforward. They are not consistent with the general increase in respiro-fermentative metabolism observed or with the increase in glycerol accumulation under the same circumstances (Chapter 2). We can speculate that the maximum expression of the correspondent gene is attained at 2.5M NaCl, or that the enzyme activity is maximized under the physiological conditions of the cells around that amount of salinity. This hypothesis will be further discussed when analysing the glycerol synthesis enzymes under stress.

Figure 4.14. Specific activity of enzymes in cell free extracts of *C. halophila* cells grown at increasing salinities with glucose as carbon and energy source. S.A. Specific activity in mU mg^{-1} protein (U = µmol min^{-1}), except for malate dehydrogenase (MLD) in which activity was expressed as U mg^{-1} protein.

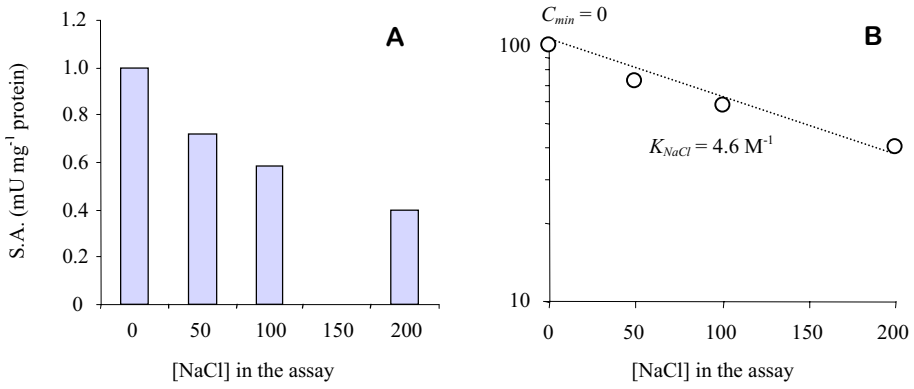


S. cerevisiae is a moderately salt tolerant yeast, although most of the studies concerning salt stress response have been performed in this well known yeast. Some of the enzymes assayed in *C. halophila* for specific activity have been reported in *S. cerevisiae* to be responsive to salt stress. TDH1, one of the forms of glyceraldehyde-3P dehydrogenase showed an increase in the protein

expression [Norbeck *et al*., 1996] when *S. cerevisiae* was shocked for 1 hour with 0.7M of NaCl. The same type of results were obtained for the mRNA expression of the genes *GLK1* [Hirayama *et al*., 1995; Rep *et al*., 2000; Posas *et al*., 2000; Yale *et al*., 2001], *HXK1*, *ENO1* [Norbeck *et al*., 1997], *PDC1*, *ALD2* [Miralles *et al*., 1995; Posas *et al*., 2000], *ALD6* [Aktar *et al*., 1997; Posas *et al*., 2000], *CIT1*, *IDH1*, *SDH1* and *MDH*, encoding glucokinase, hexokinase, enolase, pyruvate decarboxylase and aldehyde dehydrogenases, citrate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase and malate dehydrogenase, respectively. If we observe Figure 1.2 in Chapter 1, it seems that a general increase in the enzymes of glycolysis and Krebs cycle occurs, however if this increase is transient or durable it is not known. The increase in transcription of these genes should, at first hand, correspond to an increase in the general metabolic fluxes in *S. cerevisiae* identical to the one we observed in *C. halophila* though not necessarily of the same magnitude. The significance of changes in expression of all these genes in the general stress response is far from being understood. Some of these transcription responses may not correspond to physiological changes. Furthermore, some can be just *spilling energy* processes. Assays in *S. cerevisiae* were made for low salt concentrations using small periods of time, whereas the activities measured in *C. halophila* refer to cells growing actively, *i.e*., fully adapted.

The increase in activity observed in enzymes from *C. halophila* cultivated under stress should be due to a generalized transcription and/or translation regulation process leading to an increase in enzyme intracellular concentration. We wondered if, besides, *C. halophila* enzymes had also the ability to behave like halophilic enzymes. To be able to compare results with literature [Neves *et al*., 1997] we chose to assay the effect of salt on the performance of TDH. Glyceraldehyde 3-P dehydrogenase was assayed with increasing salt concentrations in the assay buffer (Figure 4.15).

Figure 4.15. A-Glyceraldehyde-3-phosphate dehydrogenase specific activity measured in cell-free extracts of *C. halophila* cultivated on 2% glucose (w/v) and collected in mid-exponential phase and assayed in buffer containing different salt concentrations. **B**-Activity presented as percentage with the determination of the NaCl exponential inhibition constant (K_{NaCl}).



Specific activity decreased exponentially with increasing salt concentrations. At 200mM NaCl the activity was reduced to 40%. The exponential inhibition constant (K_{NaCl}) determined (Figure 4.15) was smaller but still of the same order of magnitude of the one published for *D. hansenii* for the

same enzyme [Neves *et al.*, 1997]. On the other hand C_{min} was higher for *D. hansenii* (50 mM) than for *C. halophila,* which is in agreement with what has been published concerning *D. hansenii* abilities under salt stress [Prista *et al*., 1997]. Two other enzymes were assayed in *C. halophila* in the same manner as TDH, GPD (glycerol 3-P dehydrogenase) and DAK (dihydroxyacetone kinase). These reduce their activity to 32 and 38 % respectively in the presence of 500 mM NaCl (not shown). This type of sensitivity to the presence of salt have 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]. Results clearly indicate that *C. halophila* enzymic system, in contrast to halophilic and extremely tolerant bacteria, is not adapted to the presence of high ionic strength, and that most likely the intracellular ions level is quite low. Yeasts differ considerably from bacteria in the type of solutes they utilize as compatible solutes. Bacteria use preferentially amino acids and K^+ ions. Thus, their cellular components evolved to be highly adapted to the presence of concentrations such as 3M of K^+. For instance, enzymes isolated from halophilic or halotolerant bacteria like malate dehydrogenase, showed to have a different chemical structure with more acidic side chains and demonstrated to be only stable in the presence of high concentrations of K^+ and Na^+ [Cendrin *et al*., 1993; Obón *et al*., 1996; Ventosa *et al*., 1998; Sleator and Hill, 2001]. As discussed in Chapter 1, one of the strategies developed by microorganisms is the salt-in strategy, in which the intracellular environment is adapted to the presence of salt. This is not the common strategy used by yeasts, in which the cellular metabolism is dependent on mechanisms responsible for salt extrusion. The intracellular ion content of *C. halophila* cells growing in the presence of salt will be approached in Chapter 5.

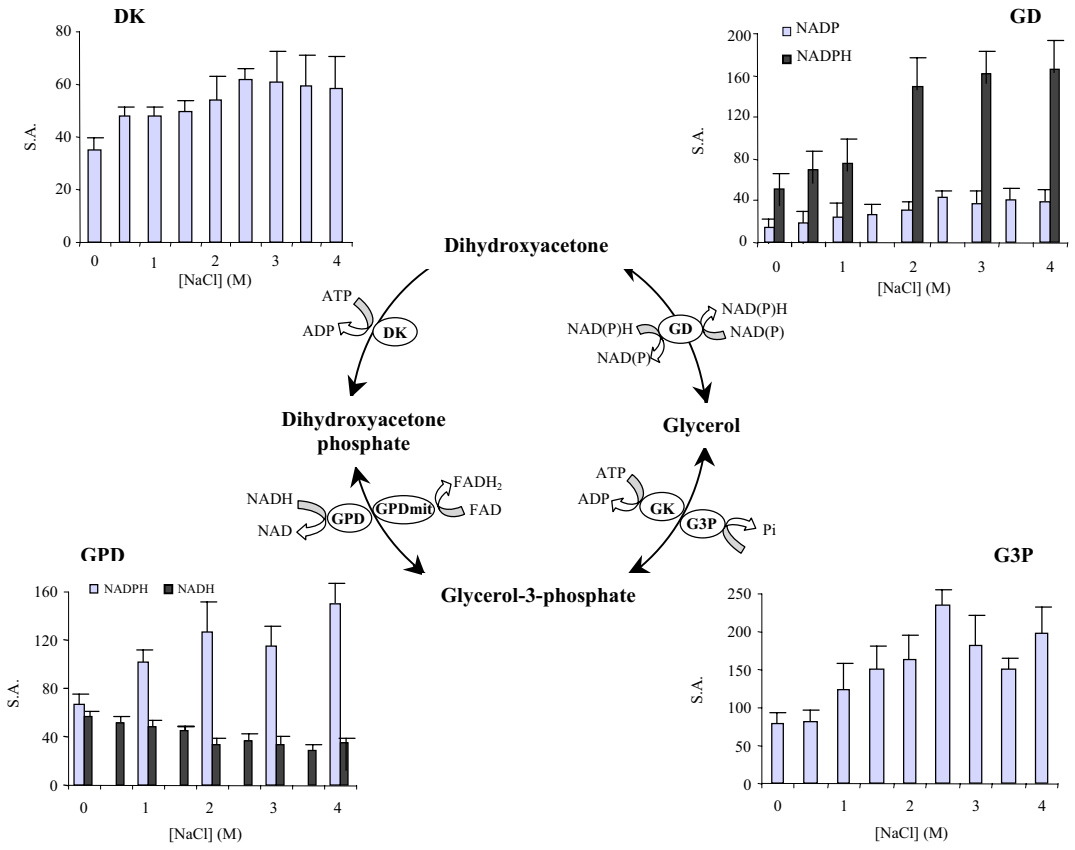
One interesting aspect of *C. halophila* metabolism is that glucose, glycerol and mannitol showed to produce the same type of general physiological "strategy" in *C. halophila* cells submitted to salt stress. As presented in Chapter 2 all these carbon sources produced the same type of performance in growth rates under stress, *i.e.*, an increase in specific growth rates up to 1.5M NaCl. We have determined the ADH specific activities in mannitol grown cells with increasing salt concentrations and verified that, as in glucose grown cells, the values increased progressively with increasing salt concentrations (an increase of about 167% was determined at 4M of NaCl- not shown).

4.2.3. *Glycerol metabolic pathways in salt-stress grown cells*

According to the results in 4.2.1, glycerol consumption in *C. halophila* appears to operate through dihydroxyacetone and not through glycerol-3P as in *S. cerevisiae* [Ansell *et al*., 1997]. Since glycerol amounts in *C. halophila* cells grown on glucose with increasing salt concentrations increase substantially according to glycerol's role of compatible solute it was expected that the enzymes activity would also change. We tested for activity the enzymes from the alternative known glycerol pathways in *C. halophila* cells thus cultivated: glycerol-3P dehydrogenase (GPD), glycerol 3-P phosphatase (G3P), glycerol dehydrogenase (GDH), dehydroxyacetone kinase (DK) and mitochondrial FAD-dependent glycerol-3P dehydrogenase (GPD_{mit}). As mentioned before glycerol kinase (GK) activity was not detected. Furthermore, GPD_{mit} was poorly detected and the values obtained do not allow conclusion as to the presence or absence of this enzyme in *C. halophila* cell-free extracts. As a mitochondrial enzyme this should most probably been assayed in mitochondrial extracts, which we did try. Results obtained are represented in Figure 4.16. *C. halophila* cells growing in the presence of salt presented activity for GPD-glycerol-3-phosphate dehydrogenase, and for G3P-

glycerol-3-phosphatase (Figure 4.16). These enzymes are constitutive and responsive to salt stress, which is concomitant with its function to accumulate glycerol under stress, similarly to *S. cerevisiae* and other yeasts (Section 4.1.6.1). Curiously, GPD accepted both cofactors although in the presence of salt only the use of NADPH as a co-factor resulted in the expected increase on specific activity of this enzyme (Figure 4.16).

Figure 4.16. Specific activities of enzymes involved in glycerol production and synthesis in cell free extracts of *C. halophila* glucose grown cells in the presence of increasing salt concentrations. S.A. expressed as U mg^{-1} protein (U = µmol min^{-1}). **DK** – dihydroxyacetone kinase; **GD** – Glycerol dehydrogenase; **GPD** – Glycerol-3-phosphate dehydrogenase; **G3P** – Glycerol-3-phosphatase



GPD enzyme has been characterized in *S. cerevisiae, Z. rouxii* and *S. pombe* as NAD-dependent, although activity for both cofactors has been reported before in *Z. rouxii* [Edgley and Brown, 1983; Ohshiro and Yagi, 1996]. In this yeast GPD presented higher activity values for NADPH as co-factor than NADH even in the presence of 1M of salt [Ohshiro and Yagi, 1996]. van Zyl and collaborators (1991)

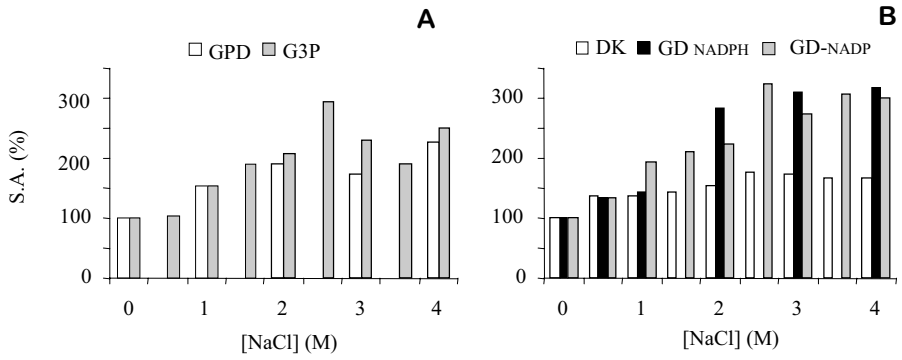
reported that GPD did not increase in the presence of 1M salt, and the other hand Ohshiro and Yagi, (1996) published an increase of 178% in specific activity in the presence of salt, not only with NADH but also with NADPH as cofactors, though the authors claimed that this increase was not significant. Regardless to co-facto utilization, GPD activity in *C. halophila,* like in *D. hansenii* [Adler *et al.*, 1985; Larsson and Gustafsson, 1987] and *S. cerevisiae* [Edgley and Brown, 1983; Blomberg and Adler, 1989] increased in the presence of salt. We cannot forget that most of the results reported for GPD response to stress have been obtained in short periods of time under stress, *i.e.*, in cells subjected to salt shock (Section 4.1.6.1.). The degree of activity changes reported can thus not be compared with the ones in this work. The utilization of different cofactors by the *C. halophila* GPD may suggest that this yeast possesses two GPD isoforms, similarly to *S. cerevisiae* being these used for different purposes. Anyway, the use of NADPH instead of NADH points to the importance of NADPH re-oxidation under stress. Nevertheless, the NADH-GPD activity is constitutive and is still detected in salt–grown cells. GPD may however be a unique enzyme accepting both NADH and NADPH as cofactors and regulated according to the NADH/NADPH ratio in the cell.

In spite that the intracellular concentration of glycerol increased linearly up to 4M NaCl, the S.A. of the enzymes responsible for its production do not follow the same increase, instead they reach a maximum value above which no further increase is observable. These results can be discussed in the light of the changes observed in specific growth rate under the same circumstances (Chapter 2). As discussed before for the glycolytitc enzymes, also the enzymes involved in glycerol metabolism reach a plateau of S.A. increase at 2.5M NaCl (taking into consideration the standard deviation). Nevertheless, glycerol production increases linearly for higher salt concentrations (Chapter 2-Figure 2.13). This way we can reason that, for NaCl $\leq$ 2.5M, S.A. increase fulfils the necessity for increased intracellular glycerol concentration, being accompanied by the increase in the enzymes of the rest of the metabolism, as seen before. In this salt-stress range, growth rate did not change significantly and even slightly increased (Chapter 2, Figure 2.8A). Above 2.5M cells have to *force* its metabolism in order to keep synthesizing glycerol. The consequence is a longer time to divide, *i.e.*, lower specific growth rates. However, the final biomass stabilized above 2.5M (Chapter 2-Figure 2.12), which may indicate that the cells are growing slower but are able to grow with success. These findings are important aspects of osmoregulation since the intrinsic levels of tolerance to salt should correlate with the metabolic regulation output of each yeast. Besides osmoregulation, glycerol is involved in other important cellular aspects such redox balancing and glycerophospholipids synthesis, which are certainly also very important under stress. Thus being, glycerol production/consumption regulation under stress can be the crossover of several cellular events determining as a whole the tolerance level of an organism.

In what regards glycerol consumption, GD and DK activity were measured in cells cultivated on glucose and salt. As before, also the specific activity of these two enzymes increased in salt-grown cells (Figure 4.16). GD was tested using either NADH or $NADP^+$. In this manner we approached this enzyme back and forward reactions. Both increased similarly with salt-stress (Figure 4.16). As to DK activity, it apparently does not increase beyond 2.5M NaCl (Figure 4.16). Nevertheless we do not consider this stabilization significant since it would most probably imply an intracellular accumulation of dihydroacetone, which was never detected (Chapter 2). The variation on S.A. found for these enzymes was compared with the one from the enzymes involved in glycerol consumption expressing both in percentage of their value in cells cultured without salt (Figure 4.17). As can be seen, the degree of variation is quite similar. This suggests that we are facing a cycle-type metabolic pathway, in spite

of glycerol accumulation verified under stress. A similar glycerol production/consumption cycle has been proposed to exist in *D. hansenii* [Adler *et al*., 1985].

Figure 4.17. Specific activity of enzymes involved in *C. halophila* glycerol metabolism expressed as percentage. **A**-enzymes from glycerol-3-phosphate pathway; **B**- enzymes from dihydroxyacetone pathway.



Glycerol metabolism in *C. halophila* is not only striking under salt stress but also when using different carbon sources. All the enzymes belonging to glycerol metabolism and which we were able to measure activity are, consistently with previous results, constitutive and enhanced in glycerol and mannitol grown cells (Table 4.2).

Table 4.2. Specific activities of enzymes from glycerol metabolism in *C. halophila* cells grown in MM with 2% (w/v) of different carbon sources. Number of independent assays between parenthesis.

	Specific activity (mU mg^{-1} protein)				
Carbon source	GPD-NADH	G3P	GD-NADPH	GD-NADP$^+$	DK
Glucose	57 ± 12 (23)	79 ± 26 (7)	52 ±19 (5)	13 ± 9 (19)	35 ± 8 (9)
Glycerol	44 ± 12 (16)	91 ± 27 (8)	138 ± 75 (6)	109 ± 32 (17)	53 ± 20 (8)
Mannitol	90 ± 25 (7)	103 ± 28 (4)	238 ± 92 (4)	228 ± 78 (8)	72 ± 2 (3)

In a similar way as the enzymes presented in Figure 4.15 for general metabolism, glycerol metabolism enzymes reach the highest activity in mannitol grown cells. Thus, carbon sources alternative to glucose seem to exert, in *C. halophila*, some type of general repression and not just a direct influence in specific enzymes in analogy to the case of glucose sin *S. cerevisiae*. Note that the same behavior applied also to the transporters characterized before in Chapter 3.

4.2.4. *Mannitol metabolic pathways*

The fast way in which *C. halophila* grows in mannitol and the fact that all enzymes have been detected with higher activity in mannitol grown cells, prompt us to unravel the enzymes involved in mannitol metabolism with different carbon sources. Similarly to the approach developed before, these were also tested in cells cultured on glucose and increasing concentrations of salt. According to the metabolic scheme proposed for mannitol production and consumption pathways in Figures 4.10, 4.11 and 4.13, and retrieved from filamentous fungi literature as mentioned, we tested activity for mannitol-1P dehydrogenase (M1PD) and mannitol dehydrogenase (MD). Both enzymes were tested using either NADPH or NADH/NAD^+. We also tried to measure mannitol-1P phosphatase, as well as mannitol kinase (as GK), without success. Fructose dependent hexokinase activity (HXK) was reported before to be similar to HXK assayed for glucose, presenting the same activity pattern under salt stress (Figure 4.14). Activity of M1PD oxidative reaction using $NADP^+$ or NAD^+ was not detected, which together with the absence of a MK may indicate that the mannitol consumption pathway through mannitol-1P (similarly to the G3-P for glycerol) is not present in *C. halophila*. Results for M1PD and MD are shown in Table 4.3. Consistently with mannitol consumption needs, and due to the absence of MK activity, NAD^+-dependent MD activity was detected and increased substantially in mannitol-grown cells. Nevertheless, since it also increased to a similar level in glycerol-grown cells, we postulate that it is regulated in the same manner as the other enzymes shown before, which maximum activity was found on mannitol grown cells, regardless of the carbon source connection. MD was detected in both directions (reduction of fructose and oxidation of mannitol), however with different cofactors specificity (Table 4.3).

According to results in Table 4.3, mannitol is probably being produced, in all carbon sources tested, through the mannitol-1P pathway, in spite that no phosphatase activity was determined. The possibility of mannitol being produced through the fructose pathway has never been proved in yeasts, only in the mushroom *A. bisporus* [Morton *et al*., 1985].

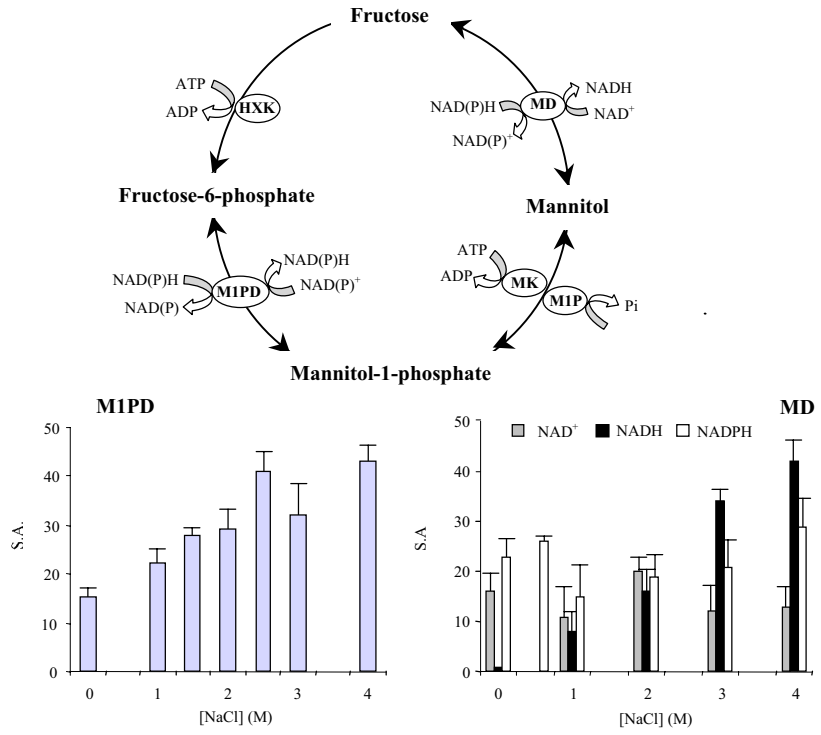
Table 4.3. Specific activities of enzymes from mannitol metabolism in *C. halophila* cells grown in MM with 2% (w/v) of different carbon sources. Number of independent assays between parenthesis. n.d-not determined.

	Specific activity (mU mg^{-1} protein)				
Carbon source	M1PD-NADPH	MPD-NADH	MD-NADPH	MD-NADH	MD-NAD^+
Glucose	15 (1)	2 (1)	23 (1)	6 (1)	16 ± 0.8 (2)
Glycerol	n.d	n.d	35 ± 8 (2)	7 ± 3 (2)	65 ± 8 (3)
Mannitol	n.d	n.d	33 ± 10 (2)	1.1 (1)	72 ± 2 (3)

The enzymes detected were further assayed in salt-grown cells, in spite that the earlier results on mannitol accumulation under stress pointed to the absence of a direct implication of mannitol in osmoregulation as a compatible solute. As can be observed in Figure 4.18 NADPH-dependent MPD increased with increasing salt concentrations up to 2.5M NaCl. We were able to detect NADH-dependent M1PD activity at 4M NaCl but the values were extremely low (4.4 mU mg^{-1} protein). The increase in S.A. of M1PD is surprising since mannitol intracellular concentration in glucose salt-grown cells decreases and is not detected above 1.5 M NaCl (Chapter 2, Figure 2.13). A possible

explanation may be that this enzyme is not specific for fructose-6P and might accept other substrates such as dihydroxyacetone phosphate, since it was assayed in similar conditions as GPD. Thus, most probably we are referring the same enzyme using either substrate and cofactor. Considering the above, we may justify the increase in specific activity under stress, since GPD activity increased for osmoregulation purposes. As expected NAD^+-dependent MD activity was not salt stress responsive and the values were constant up to 4M NaCl (Figure 4.18). However the reduction of fructose through MD in a NADPH and NADH dependent form increased under salt stress (Figure 4.18). As explained above, mannitol production via fructose is unfavourable in yeasts because, to our knowledge, fructose is not a usual intermediate in yeasts. Again, the most probable explanation is that MD can be the same enzyme as glycerol dehydrogenase, and since GD increased under salt stress conditions, what we are measuring might the enhanced expression of a unique enzyme involved simultaneously in the metabolism of glycerol and mannitol.

Figure 4.18. Specific activities of enzymes from mannitol metabolism under salt stress in glucose grown cells of *C. halophila*. (S.A. mU mg^{-1} protein; U= µmol min^{-1})



A NADPH regenerating system using the mannitol production pathway through M1P and the mannitol utilization pathway through fructose as been suggested in fungi [Hult *et al*., 1980] (Figure 4.10). This system was based in the principle of a transhydrogenase function, *i.e.*, the conversion of NADH and $NADP^+$ into NADPH and NAD^+, since yeasts do not possess transhydrogenases. These types of

cycles, as explained before, were also postulated to be ATP spending futile cycles [Blomberg, 2000], although this remains to be experimentally demonstrated.

4.2.5. *Pyridine nucleotides balance under salt stress*

During yeast growth, not only a surplus of NADH is produced due to the assimilation of sugars to biomass and to the production of various metabolic end products such as acetic acid, succinic acid, pyruvic acid and acetaldehyde, but also some sub-products like glycerol, are produced in order to re-oxidate the NADH in surplus, thus establishing a redox equilibrium. On the other hand, NADPH, mainly used in biosynthetic reactions, has to be also controlled by the cell. The absence of a transhydrogenating system that may convert NADH into NADPH, yeast cells optimised internal schemes supported by some essential metabolic enzymes such as glucose-6-phosphate dehydrogenase (G6PDH), belonging to the pentose phosphate pathway, and isocitrate dehydrogenase (IDH). These enzymes have been considered to be highly important in NADPH generation (Figure 4.12), and for this reason we decided to determine their activity in *C. halophila* grown in the absence and in the presence of salt. Both enzymes were detected in cell free extracts of *C. halophila* grown in the absence and in the presence of increasing salt concentrations (Figure 4.19). G6DH activity showed to be constant within the salt concentrations tested, while IDH increased for salt concentrations above 2M (Figure 4.19).

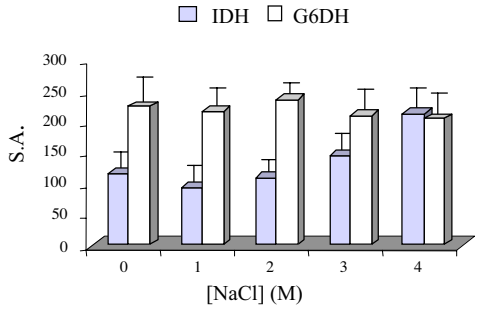


Figure 4.19. Specific activity of isocitrate dehydrogenase (IDH) and glucose-6-phosphate dehydrogenase (G6DH) in cell free extracts of *C. halophila* grown on glucose under different osmotic conditions. S.A. expressed in mU mg^{-1} protein (U = µmol min^{-1})

There is also another enzyme, the $NADP^+$-dependent acetaldehyde dehydrogenase (Ald6p) that has been reported in *S. cerevisiae* to be important in the generation of NADPH. Although increasing acetic acid concentrations were measured in cells grown in the presence of salt, we were not able, as said before, to detect any acetaldehyde dehydrogenase activity. Considering the results obtained with G6DH and IDH, it may be suggested that NADPH production is being made under stress mainly through the IDH activity and probably also by the acetaldehyde dehydrogenase. However, since we were not able to measure Aldp activity, we do not know if it is using NADH or NADPH as cofactor. G6DH seems to be accounting for the production of NADPH in *C. halophila* in a constant way in the presence and in the absence of salt stress, which may suggest that pentose phosphate pathway does not play, apparently, a special function under stress, at least in the growth conditions tested. The increase observed in IDH must be interpreted carefully, since most of the

enzymes assayed showed their S.A. increased under stress. This feature seems to be intrinsic to the metabolism of *C. halophila* cells under stress. Thus, we have no means to known, with the few results available, if IDH S.A. increase is due to a necessity to increase the net production of NADPH or if is the result of a general increase in *C. halophila* metabolism performance under stress.

In order to better understand *C. halophila* redox balancing under stress we determined the intracellular concentrations of the reduced and oxidized pyridine nucleotides in cells cultured in the presence and in the absence of stress (Table 4.4). Cofactors concentrations did not vary significantly in the presence of salt up to 4M, being slightly reduced in the presence of salt when compared with results obtained in the absence of salt (Table 4.4).

Table 4.4. Intracellular concentration of NAD^+, NADH, $NADP^+$ and NADPH in *C. halophila* cells under different osmotic conditions when using glucose. **P**hosphorylated **N**ucleotide **F**raction (PNF) = $((NADP^+ + NADPH)/(NAD^+ + NADH))$; **C**atabolite **R**eduction **C**harge (CRC) = $(NADH/(NAD^+ + NADH))$; **A**nabolic **R**eduction **C**harge (ARC) = $(NADPH/(NADP^+ + NADPH))$. Results are the mean of at least three independent assays.

	Intracellular pyridine nucleotide concentrations (μmol g^{-1} d.w.)								
[NaCl](M)	NAD^+	$NADP^+$	NADH	NADPH	NAD^+/NADH	$NADP^+$/NADPH	PNF	CRC	ARC
0	1.44	0.44	1.97	1.18	0.73	0.37	0.48	0.49	0.73
0.5	0.87	0.15	1.14	0.71	0.76	0.21	0.43	0.57	0.83
1.0	0.94	0.15	1.04	0.81	0.90	0.19	0.49	0.53	0.84
1.5	1.18	0.30	0.81	0.85	1.46	0.35	0.58	0.41	0.74
2.0	0.96	0.29	1.14	0.93	0.84	0.31	0.58	0.54	0.76
2.5	1.22	0.18	1.14	1.02	1.07	0.18	0.51	0.48	0.85
3.0	0.91	0.38	1.04	0.89	0.88	0.42	0.65	0.53	0.70
4.0	0.52	0.18	1.65	1.10	0.35	0.16	0.59	0.76	0.86
Mean + SD.	1.01 ± 0.26	0.26 ± 0.10	1.24 ± 0.35	0.93 ± 0.15	0.87 ± 0.31	0.27 ± 0.10	0.54 ± 0.07	0.54 ± 0.10	0.79 ±0.10

Results obtained strongly suggest that *C. halophila* is controlling efficiently the redox balance under heavy salt stress, which corroborates the growth performance presented in the previous chapters. This way the indexes PNF, CRC and ARC maintained inevitably constant. The increases observed previously in ethanol, glycerol and acetic acid production under stress are most certainly connected with this equilibrium achievement. Studies concerning pyridine nucleotides are rather scarce. Most of them correspond to studies using cells growing actively under optimal conditions and thus in a biochemical steady-state. It is thus natural that no substantial differences in co-factors concentrations are found. This is especially true in what regards the balance between the two types of co-factors, taking as a bulk their oxidized versus their reduced forms. This has been shown in *A. niger* cells during citric acid fermentation [Führer *et al*., 1979]. Pyridine nucleotide concentrations decreased progressively through the fermentation process, but the ratio $(NADP^+ + NADPH)/(NAD^+ + NADH)$, representing the phosphorylated nucleotide fraction (PNF) remained constant. This was not the case of *C. halophila* growing under stress.

Results concerning the intracellular pyridine nucleotide concentrations of several

microorganisms, including those of *C. halophila* obtained in the absence of salt in this work, were joined in same Table for comparison (Table 4.5). As can be seen, in spite that all results were obtained in batch cultivations, each nucleotide has a specific level in each microorganism (Table 4.5).

Table 4.5. Pyridine nucleotides levels and ratios in different microorganisms (μmol g^{-1} d.w.)

Nucleotides	*Escherichia Coli*[a]	*Pseudomonas sp*[b]	*Aspergillus niger*[c]	*Penicillium chrysogenum*[d]	*Saccharomyces cerevisiae*[e]		*Candida halophila*[f]
					aerobic	*anaerobic*	
NAD^+	8.6 ± 2.0	3.50	1.70 ± 0.25	3.15 ± 0.45	1.50	2.87 ± 0.09	1.44
NADH	1.1 ± 0.2	0.19 ± 0.05	0.18 ± 0.03	0.13 ± 0.02	1.81	0.44 ± 0.01	1.97
$NADP^+$	1.0 ± 0.2	0.27	0.14 ± 0.02	0.16 ± 0.02	0.04	0.23 ± 0.01	0.44
NADPH	1.3 ± 0.3	0.80 ± 0.18	0.08 ± 0.02	0.09 ± 0.02	0.21	1.21 ± 0.07	1.18
NAD^+/NADH	7.8	18	9.4	24	0.83	6.5	0.73
$NADP^+$/NADPH	0.77	0.34	1.75	1.78	0.19	0.19	0.37
PNF	0.24	0.29	0.12	0.08	0.07	0.44	0.48

[a] Values from Andersen and von Meyenburg, 1976; [b] Values from Matin and Gottschal, 1976; [c] and [d] Values from Führer *et al*., 1979; [e] Aerobic values from Sáez and Lagunas, 1976, Anaerobic Nissen *et al*., 2001; [f] Results from the present work.

Data obtained for the different microorganisms (Table 4.5), support the idea that NAD(H) nucleotides are largely present in the oxidized form (the ratio NAD^+/NADH values are greater than the unit) except for *S. cerevisiae* under aerobic conditions and for *C. halophila*, whereas the NADP(H) ratio varies in each organism. It is noteworthy to stress that *C. halophila* has a high NADPH level when compared with the other microorganisms (Table 4.4). This aspect is very important since glycerol production under stress is NADPH dependent and thus the NADPH consumption under stress for osmoregulation purposes may lead to a lower availability of NADPH, being the NADPH-dependent processes such as biomass production compromised. In fact, above 2.5M NaCl, growth rate is decreasing progressively (Chapter 2), although final biomass stills high, being their value only dependent on the pH media. The high levels of NADPH are intriguing in *C. halophila* metabolism, since it appears that this yeast is somehow producing an NADPH surplus. It is possible that *C. halophila* dissimilates normally part of the glucose through the pentose phosphate pathway, leading to an extra NADPH production. If this is the case, then all metabolism will have to be balanced with reactions consuming NADPH. It is obvious that only a metabolic flux analysis will be able to test this possibility. The use of a respiro-fermentative metabolism is advantageous for redox balancing, since respiration allows the recycling of the cofactors through the mitochondria, and thus the increase in acetate and ethanol production may be no more than the result of the general pattern of metabolism stimulation observed under stress and not a directly involved in redox balancing.

In spite of the central importance of the cofactors in microorganisms metabolism, in practice there is still limited knowledge in the redox balance mechanisms, and although the cofactors measurements in *C. halophila* confirm a balanced metabolism under heavy stress they are without any comparison. Recently it was reported an important study in *S. cerevisiae* that correlates life span, calorie restriction, high osmolarity and NAD^+/NADH ratios [Kaeberlein *et al*., 2002]. Lifespan in the

budding yeast *S. cerevisiae* is extended by a variety of stimuli such as heat stress, osmotic stress and the restriction of amino acids or glucose availability [Lin *et al*., 2000; Swiecilo *et al*., 2000; Kaeberlein *et al*., 2002]. The latter two regimens are considered to be mimics of calorie restriction in higher organisms. Calorie restriction has been proved to extent greatly the life span of many diverse organisms. In *S. cerevisiae* calorie restriction extends lifespan by increasing respiration [Lin *et al*., 2002] in a process that requires NAD^+ and the silent information regulator gene *SIR2,* which encodes for NAD-dependent histone deacetylase activity [Lin *et al.*, 2000], and is governed by nicotinamide and the gene *PNC1,* which encodes an enzyme that deaminates nicotinamide [Anderson *et al*., 2003]. These authors reported recently evidences that Pnc1p regulates Sir2p by modulating intracellular nicotinamide. The same authors do not exclude the possibility that Sir2p may be regulated passively together with nicotinamide depletion, according to changes in either NAD^+ availability or the NAD^+/NADH ratio. Kaeberlein and collaborators (2002) reported that high osmolarity extends the lifespan of *S. cerevisiae* by activating Sir2p through a likely increase in NAD^+ levels. In fact, genetic and microarray analysis indicate that high osmolarity extends the lifespan by activating Hog1p, which in turn leads to, between other processes, an increase in the biosynthesis of glycerol from glycolytic intermediates. This metabolic shift likely increases NAD^+ levels, thereby activating Sir2p and promoting longevity.

If we look carefully to the NAD^+/NADH ratios on Table 4.4, a slight progressive increase in the values from 0 to 1.5M NaCl is apparent, though it was not considered significant. Actually, 1.5M NaCl was the salt concentration leading to the highest *C. halophila* μ_g value (Chapter 2). This way, we might postulate that the NAD^+/NADH ratios are one of the factors controlling/allowing/modulating salt stress resistance in *C. halophila* as in other microorganisms. Furthermore, taking into consideration that fermentation rate is substantially enhanced in cells growing at ≥2.5M NaCl, at which μ_g value decreases steeply, we can speculate the expansion of *C. halophila* life span. It duplicates slower but it survives metabolically, by one side producing enough glycerol to osmoregulate and on the other side increasing intracellular NAD^+ concentration.

Obviously, we cannot apply directly the knowledge in *S. cerevisiae* to *C. halophila*, since they are quite different yeasts. However, the hypothesis concerning the involvement of a nuclear NAD^+/NADH ratio directly in the metabolism and consequently in the life span of microorganisms, highlights the importance of certain less studied physiological aspects. We should keep in mind that co-factors ratios might not be too important for metabolism regulation as long as they are turning-over. They might be determinant once one particular form accumulates creating a kind of metabolic "jam". From all above exposed we conclude that only an integrated view of enzymes activities, intracellular concentrations of intermediates and by-products, and the evaluation of the ratios NADH/NAD^+ and NADPH/$NADP^+$ can provide a picture of the intracellular state of an organism when cultivated in a specific medium and a specific cultivation system.

4.3. Concluding remarks

The enzyme specific activities determined in *C. halophila* cells grown under salt-stress and in diverse carbon sources within this chapter confirmed results obtained previously on growth rates variation, respiration and fermentation rates and metabolites production. They have shown that the metabolism of *C. halophila* is substantially and generally improved under salt stress. From the results presented we can highlight the following:

- A general pattern of enzyme activity stimulation in cell cultures under increasing salt-stress was observed (with few exceptions) suggesting a general pattern of transcription/translation stimulation by salt.
- Activity stimulation of enzymes from glycolysis/Krebs cycle results is compatible with the maintenance of respiro/fermentative metabolism under stress, and, in particular, with the increase observed in fermentation rates in these circumstances.
- All enzymes assayed and for which we were able to determinate a specific activity were constitutively expressed in glucose, glycerol, mannitol and ethanol grown cells, although subjected to some degree of regulation.
- Mannitol was the carbon source producing the highest increase in specific activities, an effect already observed in the kinetic parameters of the transporters characterized in the Chapter 3.
- Glycerol and mannitol kinase activity were not detected in any of the carbon sources tested, neither was GDH_{mit}. This suggests that the correspondent pathways of glycerol and mannitol dissimilation are not present in *C. halophila*.
- The results clearly show that glycerol production under salt-stress is performed via glycerol 3-P like in *S. cerevisiae*.
- Glycerol-3P dehydrogenase revealed the ability to use either NADH or NADPH as co-factors but used NADPH as preferred co-factor in salt-growing cells.
- Glycerol dehydrogenase and dihydroxyacetone kinase activity were determined suggesting glycerol consumption operates through dihydroxyacetone pathway.
- We cannot disregard the possibility of dihydroxyacetone pathway being alternatively active for glycerol synthesis, though it cannot be determined with certainty. Glycerol dehydrogenase activity was determined in both glycerol consumption and synthesis directions, but we could not determine dihydroxyacetone phosphatase activity.
- Mannitol was synthesized through the mannitol-1-phosphate pathway, in spite of the absence of mannitol-1-phosphatase activity detection. On the other hand mannitol was dissimilated, similarly to glycerol through a mannitol dehydrogenase followed by action of a fructose-dependent hexokinase.
- Strangely mannitol-1-phosphate dehydrogenase activity increased in the presence of NaCl, which is not in accordance with the findings exposed in Chapter 2, in which mannitol accumulation under stress decreased steeply, being not detected $\geq$ 2M NaCl. The logical explanation seems to be the involvement of mannitol in a futile cycle simultaneously with a function in redox balance.
- Mannitol was the carbon source enhancing the highest increase in specific activities, an effect already observed in the kinetic parameters of the transporters characterized in the Chapter 3, which may suggest that mannitol has an important function in *C. halophila* metabolism.
- Cofactors intracellular measurements showed an equilibrated balance under stress.