Chapter 3

Sugar and polyol transport characterization

3.1. ***Introduction***

3.1.1. *Cell wall, plasma membrane and cytoskeleton composition in yeasts*

3.1.2. *Regulation of yeasts cell volume under salt stress*

3.1.3. *Plasma membrane transport mechanisms in yeasts*

3.1.3.1. *Glucose transport*

3.1.3.2. *Polyol transport*

3.2. ***Results and discussion***

3.2.1. C. halophila *cell volume under salt stress*

3.2.2. *Transport systems in* C. halophila

3.2.2.1.*Glucose uptake study*

3.2.2.2. *Glycerol uptake study*

3.2.2.3. *Mannitol uptake study*

3.3. ***Concluding remarks***

Summary

Usually the first step in any compound metabolism is its transport across the plasma membrane. This way, transport can be a crucial step in proliferation. The most frequent types of transport systems in yeasts are facilitated diffusion and active transport. In this chapter we present the transport characterization, as complete as possible, of compounds accumulated intracellularly by *C. halophila* cultivated both in the absence and in the presence of salt. The transport characteristics of glycerol, mannitol and glucose uptake were determined in cells grown in synthetic medium with glucose, ethanol, glycerol or mannitol as single carbon and energy sources in batch system. Furthermore, these compounds transport was also assayed in cells cultured in glucose and increasing salt concentrations. The results obtained suggest that glucose is transported by a facilitated diffusion mechanism which *Vmax* values decrease on cells cultivated in the presence of salt accompanying the decrease of the specific growth rate of the same cultures, and indicating that glucose transport might be the rate limiting step for growth under such circumstances. Glycerol was shown to be actively transported through a proton symport as confirmed by this substrate uptake sensibility to drugs affecting p.m.f. as well as the intracellular accumulation ratio against chemical gradient. Yet, no glycerol initial uptake velocity/initial alkalinization rate stoicheiometry could be determined. On the other hand, according to identical results as the ones obtained for glycerol uptake, mannitol appears to be actively transported, though no external alkalinization upon mannitol addition to a cell suspension could be detected with cells obtained in all growth conditions mentioned. Finally, both glycerol and mannitol transport *Vmax* decreased for cells cultivated in increasing NaCl concentrations and were higher in cells cultivated in carbon sources other than glucose. In spite of being under regulation by carbon source and stress, glucose, glycerol and mannitol transport systems were found to be constitutively expressed.

3.1. Introduction

The yeast membrane transporters play crucial roles in functions as diverse as nutrient supply, drug resistance, salt tolerance, control of cell volume, efflux of undesirable metabolites and sensing of extracellular nutrients [van Belle and André, 2001]. They also provide essential constituents of energy-generating and energy-consuming systems [Pao *et al*., 1998].

A major breakthrough in the study of transport in yeasts is the discovery that some transporters homologues function as sensors of external nutrients. The main challenge for future studies on transport in yeast will be to characterise transporters predicted in genome analysis but whose function remains unknown and understand how transporters are regulated in order to ensure homeostasis of the many compounds present in the cell.

3.1.1. *Cell wall, plasma membrane and cytoskeleton composition in yeasts*

Cell wall

The outermost layer of the yeast cell envelope is the cell wall, an essential organelle in fungal species representing up to 30% of the dry weight of the cell [Klis, 1994; Stratford, 1994]. The yeast cell wall has many functions: physical protection, osmotic stability, enzyme support, binding of compounds, cell/cell adhesion and selective permeability barrier [Stratford, 1994]. Cell wall is a layered structure freely permeable for solutes smaller than 600 Da and in *S. cerevisiae* consists basically of three major representative polysaccharides: (i) *ß*-glucans (47 to 60% of the wall weight), (ii) chitin (0.6 to 9%) and (iii) mannoproteins (25 to 40%). In addition, cell walls contain proteins (6 to 25%) and minor amounts of lipids (1 to 7%) [de Nobel *et al*., 1989, 1990 a,b]. The internal layer of *ß*-glucan determines the rigidity of cell wall, and chitin represents the structural components of the cell wall [de Nobel *et al*., 1990 a,b]. Together they form a rigid skeleton that provides strong physical properties to the cell and plays very important roles in determining cell morphology, cell division, and osmotic stability of an organism, all of which are critical for survival of the yeast in its permanent changing natural environment [Bacon *et al*., 1964, Cabib *et al*., 1988; Hong *et al*., 1994]. Although chitin is a minor component in *S. cerevisiae* cell wall it is important in budding scars. The external layer of mannoproteins determine the porosity of the cell wall and are the main material of the cell wall matrix representing 40% of the total cell wall polysaccharide [de Nobel *et al*., 1990 a,b]. The different cell wall components, though not evenly distributed, interact with each other to give rise to the overall architecture of the cell wall, turning it an important flexible layer, namely in stress conditions which impose serious damage to cells. Cell wall structure is dependent of many conditions, such as growth medium composition, temperature and growth phase and even the type of strain used [de Nobel *et al*., 1990 a,b]. Among the yeasts already studied such as *C. albicans*, *S. pombe*, *D. hansenii*, *Kloeckera apiculata*, *Z. bailii*, *Z. rouxii, Kluyveromyces marxianus* and *S. cerevisiae*, cell wall composition differs to some extent, between strains of the same species and thus need to be studied independently [Cottrell *et al*., 1986; Ishiguro, 1998; Nguyen *et al*., 1998].

Cell wall composition in salt stress conditions have been poorly studied and the few results obtained are not conclusive. For instance, Hamada and collaborators (1984) studied the composition of the cell wall in *Z. rouxii* cells growing in the absence and in the presence of 2.6 M of NaCl. They did not find significant changes between the composition of the cell wall in either cells, however, in the presence of NaCl the side chains consisting of a mannobiose unit where slightly reduced.

Although the structure of certain mannans revealed to be different from the ones of the non-salt-tolerant yeast *S. cerevisiae*, it was not clarified whether the difference in the chemical structure of cell wall mannans had any relation with the mechanisms of salt tolerance of the yeasts. In the same way Tomita and collaborators (1996) studied in *Z. rouxii* the influence of salt in the cell wall chitin composition. Once again, although they observed clearly that chitin content diminishes with the increase in NaCl in the medium and that this was a characteristic of this yeast, it was not possible to relate that observation to the halo-tolerant abilities of this yeast. More recently, Sengar and collaborators (1997) observed that a MEK (MAKK) kinase -Mkh1 from *S. pombe* is required for cell wall integrity and proper response to osmotic stress. Cells deleted in *MKH1* gene required a longer time to re-enter the cell cycle after a prolonged stationary phase arrest and exhibited a round shape instead of an elongated shape. Besides, their growth in the presence of NaCl or KCl was inhibited. Dubois and collaborators (2002) found very recently that Kcs1 kinase is involved in inositol signalling, sustained cell growth, biogenesis of the vacuole, integrity of the cell wall and promoted adaptative responses to salt stress. Thus, studies point to the importance and the relationship between cell wall, salt stress resistance and the normal functioning of cells. However, the osmotic effect in yeast´s cell wall is known for long time with the studies of Schwencke and collaborators (1971) and Morris and collaborators (1986) who reported that cells submitted to osmotic stress (provoked by 1.5 M of mannitol and by sorbitol) have its porosity lowered in the same range as the cell volume. In spite of many cell wall compounds being assigned as quite important in yeast cellular functions, its biophysical and biological functions, namely in stress conditions, still needs to be further studied.

Plasma membrane

Besides cell wall, yeasts possess a plasma membrane or cytoplasmic membrane, which forms a relatively impermeable lipid bilayer barrier for hydrophilic molecules. Membranes are matrices into which many functional proteins are embedded and maintained in an interfacial position. The dynamics and structural characteristics of membranes turn it very sensitive to changes in environmental conditions, being easily disorganized. Thus, in order to benefit from its vital function, cells need to maintain membrane structure and dynamic properties compatible with cell integrity [Beney and Gervais, 2001].

Plasma membrane is a bilayer constituted by a mixture of polar lipids and proteins, which, by their interactions, govern the structure of the membrane [van der Rest *et al*., 1995]. There are several types of proteins, which are distributed asymmetrically on the membrane, such as cytoskeleton proteins, cell wall synthesis and repair proteins, signal transduction related proteins and transport proteins [van der Rest *et al*., 1995]. The lipids are also disposed asymmetrically across the bilayer and are very diverse in size and composition. The lipid composition of the plasma membrane is complex and tightly regulated, suggesting that lipids play a role in the activity of the proteins in the plasma membrane [van der Rest *et al*., 1995]. Changes in the environment induce variations in the membrane fluidity, and even in the membrane lipid composition. Changes in the membrane's structural and dynamic characteristics affect the functions of membrane proteins by changing the lipid environment of enzymes, and may also be involved in the cellular response to stress [Beney and Gervais, 2001].

Fluidity and structure order are two important characteristics of plasma membrane, which are relevant in the physiological adaptation to environmental stresses and are given essentially by the plasma membrane composition in fatty acid chains [Swan and Watson, 1997]. Fluidity is a subjective term

that describes the relative diffusional motion of molecules within membranes and is used in preference to viscosity [Laszlo *et al*., 1998]. During adaptation to environmental changes, the control of membrane fluidity is an essential functional necessity for the cell. Fluidity changes are signals which can, for example, direct mechanosensitive channels functioning or influencing the sensing by a signal transduction pathway sensor or even influence transporters ability to keep working [Beney and Gervais, 2001]. In the case of extreme or abrupt variations, the structural effects causing physical changes in the lipid organization with consequences in fluidity may not be enough to fully compensate a certain stress situation. This way, active adaptations take place, and the lipid composition of the cell membrane changes. Survival may be a balance between the fast working changes and the long-term adaptation abilities of each cell at membrane level.

When an organism grows in media containing NaCl it has frequently to undergo some physiological adaptations, such as accumulating osmolytes or actively extruding ions. In order to be able to retain a desirable high concentration of solutes, it is generally accepted that some adaptations to increased salinity and consequently osmolarity of their environment are mediated also by changes in the membrane properties, specially in bacteria where there is much more knowledge about membrane adaptations under stress [Russell *et al.*, 1995]. It is expected that the plasma membrane might become more rigid in order to retain more osmolyte within the cells for the purpose of increasing the internal osmotic pressure. In bacteria, the adaptation to growth in different salt concentrations is often reflected in changes in the composition of the membrane lipids, namely fatty acids and phospholipids, being such alterations related with the resistance level of each bacteria [Ventosa *et al*., 1998]. The most obvious adaptation strategy in bacteria involves an increase in the proportion of anionic over zwitterionic phospholipids [Sleator and Hill, 2001]. This structural modification adds additional negative surface charge to the membrane, which helps to maintain hydration of the interface and has a pronounced effect on lipid phase behavior [Sleator *et al*., 2001]. Besides bacteria, it is well known that the membrane permeability of the green alga *Dunaliella tertiolecta* as also the *Dunalliella* species, to their osmolyte (glycerol) is very low in the presence of salt, suggesting that the quantities lost by leakage through the membrane are negligible and that cell undergoes membrane salt-dependent changes in order to better adapt to stress conditions [Goyal *et al*., 1986; Hocking, 1988]. Among yeasts there are substantial differences in membrane composition and in membrane response to stress, although, some authors claim that the differences between fatty acyl profile of tolerant and non-tolerant yeasts may reflect differences in general metabolic pattern rather than bearing any direct relation to osmotolerance [Tunblad-Johansson *et al*., 1987].

Sterols are membrane components of eukaryotic biological membranes that play an important role by modulating the fluidity (rigidity) to a large extent of the plasma membrane, which, in turn may affect the lateral movement and the activity of membrane proteins [van der Rest *et al*., 1995; Daum *et al*., 1998]. Studies on halophilic black yeasts suggested that sterols synthesis in the presence of salt differs from less tolerant yeasts such as *S. cerevisiae*, which may be either related or a consequence of the different ecophysiology of halophilic yeasts [Petrovic *et al*., 1999; Turk *et al*., 2004]. Cells of *Z. rouxii* [Watanabe and Takakuwa, 1984, 1987], *C. albicans* [Combs *et al*., 1968] and *D. hansenii* [Tunblad-Johansson *et al*., 1987], when exposed to high salt concentration showed a decrease in fatty acid insaturation, which suggest the increase of the membrane rigidity (decrease in fluidity), although the content of phospholipids was almost constant till 2M of NaCl, decreasing slightly at 3 M indicating that the phospholipids content is probably little affected in halotolerant yeasts. In contrast, Khaware and collaborators (1995) observed in *C. membranefaciens* an increase in fluidity as a result to

exposure to 1.35 M of NaCl. Nevertheless, cells of *Z. rouxii* growing in the presence of 2.6 M NaCl presented three times more ergosterol than cells cultivated without salt [Hosono, 1992], which confirms the decrease in fluidity, since sterol content is for long time associated with the membrane fluidity state. Furthermore, Yoshikawa and collaborators (1995) also realized that lipids saturation seems to be important for salt tolerance of *Z. rouxii* under salt stress. Actually, the sterol-to-phospholipid ratio is considered to be a determinant attribute of membrane fluidity in eukaryotic organisms. In the halophilic melanized yeast-like fungi *H. werneckii* and *Phaeotheca triangularis*, this ratio was maintained at a level considerably lower than the halotolerant yeast-like fungi *Aureobasidium pullulans* [Turk *et al*., 2004], the salt-sensitive *S. cerevisiae* [Turk *et al*., 2004] and the halotolerant yeasts *D. hansenii* [Tunblad-Johansson *et al*., 1987] and *Z. rouxii* [Hosono, 1992]. Thus, a low sterol-to-phospholipid ratio appears to be an important characteristic for halotolerance. Due to a low sterol-to-phospholipid ratio and increased unsaturated fatty acids, it was shown that the membrane of the halophilic melanized yeast-like fungi, isolated from salterns, were more fluid than those of the halotolerant yeast-like fungi and than *S. cerevisiae* over a wide range of NaCl concentrations [Turk *et al.*, 2004]. Together, these results not only have confirmed that membrane fluidity is of crucial importance for tolerance against salt stress, but also demonstrated that higher salt tolerance correlates well with higher membrane fluidity and that the ability of microorganisms to maintain their membrane fluidity over a wide range of salinities is linked directly with halophily [Turk *et al.*, 2004]. Obviously, alterations in the composition of cytoplasmic membrane will have direct influence in solutes transport, namely osmolytes which are essential to osmotic acclimation, and may even justify the slow growth rates presented by microorganisms when growing in salt rich media.

Cytosqueleton

Nearly all eukaryotic cells contain a cytoskeleton, made up in part of microtubules and microfilaments, consisting primarily of the proteins tubulin and actin, respectively [Zubay, 1993]. Microfilaments have been shown to form bundles near the plasma membrane and are believed to have an important role in local or general changes in shape of the cell surface and thus in the plasma membrane and also function to direct cell growth to the emerging bud. According to the literature, it seems to be well established that actin cytoskeleton is involved in regulation of cell wall synthesis and hence in the control of cellular morphology [Adams and Pringle, 1984; Ayscough and Drubin, 1998]. Furthermore, Choi and collaborators (2000) suggest also a role for TOR pathway signaling in regulating microtubule stability and function, connecting again the cytoskeleton functions with regulation of cells nutrient status (regulated by the TOR signaling pathway) and cell division. Thus being, stress conditions which impose several difficulties to cells survival, should be accompanied by alterations in cytoskeleton properties. Indeed, Chowdhury and collaborators (1992) observed that osmotic stress caused a change in the actin filament organization, namely its disassembly, which leads to a temporary growth arrest. This behavior is though reversible, being more or less rapid according to the osmotic stress severity. Brewster and Gustin (1994) observed that the HOG pathway is required for repositioning the actin cytoskeleton and the normal spatial patterns of cell growth after recovery from osmotic stress, which reinforces the importance of this pathway and cytoskeleton proper function in osmotic stress response. Recent studies observed that phosphoinositides, which are ubiquitous components of eukaryotic membranes, are critical regulators of the actin cytoskeleton and membrane trafficking and also that inositol polyphosphate 5-phosphatases are involved in the

regulation of actin cytoskeleton reorganization following hyperosmotic stress [Ooms *et al*., 2000]. The disassembly of cytoskeleton and growth arrest after osmotic stress, also suggest a tight connection with cell cycle, since it is reseted some time after an osmotic shock [Chowdhurry *et al*., 1992].

Hyperosmotic shock not only induces changes in cytoskeleton but also induces changes in the organization and ultrastructure of the cell wall, apparently resulting from the displacement of periplasmic and cell wall matrix material into deep invaginations of the plasma membrane created by cell shrinkage [Morris *et al.,* 1986; Slaninová *et al*., 2000]. The deep invaginations formed during hyperosmotic stress are complementary to bulges formed on the inner surface of the cell wall [Slaninová *et al*., 2000]. Since cytoskeleton is responsible for the support and elasticity of the cell, it is not surprising that osmotic conditions will have an deep impact in this structure and consequently in cell morphology given essentially by the cell wall. This is specially true because yeasts do not exhibit typical signs of plasmolysis during hyperosmotic shock [Morris *et al.,* 1986], but they reduce their volume, meaning that cell wall and plasmatic membrane must accompany this process, resulting in the invaginations observed by some authors.

In spite of the progress made for unveiling the signal transduction pathways related with osmotic stress response, very little attention has been devoted so far to the immediate morphological and physiological effects of hyperosmotic shock in yeast cells. So far, the results have been obtained using the widespread non-tolerant yeast *S. cerevisiae* with low concentrations of solutes (0.7-1.0M of KCl or glycerol) and short periods of time [Morris *et al.,* 1986]. In *S. cerevisiae* it was seen that the period of arrest lasted about 2 h after the transfer of yeast cells to a medium containing 1M of KCl, thereafter, the cells resumed growth albeit at a slightly reduced rate. During the period of growth arrest, the cells exhibited morphological changes observable at the cellular and ultrastructural level as pointed above [Morris *et al.,* 1986]. No doubts that further studies are needed, namely using more resistant yeasts growing actively in high salt concentrations.

3.1.2. *Regulation of yeast cell volume under salt stress*

How does a cell control its size? There are two schools of thought: either a cell divides after it reaches a certain critical cell size, or cell growth and proliferation are regulated independently, with cell size emerging from a simple correlation of the two [Rupes, 2002]. Whatever the hypothesis is the most correct, it seems quite obvious that cell volume, cell growth and proliferation are related events and are tightly coordinated in any environment, in order to proportionate cell's survival. Owing to the uncertain supply of nutrients in the wild, the yeast cell-division rate must be coordinated with widely variable rates of cell growth otherwise cells would get progressively smaller or larger. Indeed there are studies which suggest that critical cell size is an prerequisite for progression through the cell cycle [Philips *et al*., 1997].

Despite their rigid cell wall, yeasts show volume changes upon osmotic shock [Morris *et al*., 1986; Meikle *et al*., 1988]. However, cells do not respond equally to osmotic shifts, being dependent on their physiological state, *e.g*., exponentially growing cells are more sensitive to osmotic shock than stationary phase cells [de Marañon *et al*., 1996]. Exposure of cells to high environmental osmolarity leads to dehydration and cell shrinking as a consequence of rapid and instant loss of the cytoplasm water, with deep impact in cells structure and in their normal metabolic functioning [Morris *et al*., 1986]. During osmotic dehydration of eukaryotic cells, the organelles will also be exposed to hypertonic solutions and will, in turn, undergo shrinkage. Re-hydration occurs as soon as the cell is exposed to normal

isotonic conditions [Morris *et al*., 1986]. Cell volume recovery has not been so extensively studied as shrinkage. This way it is not well known how the cell regains the cytoskeleton/wall organization allowing volume increase. Equally, it is not known to what extent recover is possible or, better, to what extent shrinkage is irreversible, after prolonged exposure to hypertonic environmental conditions.

The process of shrinking after a hyper-osmotic pulse made by addition of, for instance NaCl to the medium, takes less than a minute [Meikle *et al*., 1988; Vindeløv and Arneborg, 2002]. The cell volume attained correlates inversely with the concentration of the solute causing osmotic stress [Meikle *et al*., 1988; Albertyn *et al*., 1994a,b]. This correlation as been observed in different yeasts such as *S. cerevisiae* [Morris *et al.,* 1986; Reed *et al*., 1987; Meikle *et al*., 1988; Albertyn *et al*., 1994a,b; de Marañon *et al*., 1996; Lages and Lucas, 1997], *D. hansenii* [Reed *et al*., 1987; Prista *et al*., 1997; Larsson *et al*., 1990], *Z. rouxii* [van Zyl *et al*., 1993; Yagi and Nishi, 1993], *P. sorbitophila* [van Eck *et al*., 1993; Lages and Lucas, 1995], *H. anomala* [van Eck *et al*., 1989], *Y. lipolytica* [Andreishcheva *et al*., 1999], *C. cacoi*, *C. magnoliae* and *Z. bisporus* [van Eck *et al*., 1993]. Usually, the higher the solute concentration in the medium the smaller the cell size attained. *S. cerevisiae* shocked with 0.6, 0.9 and 1.2 M of NaCl reduces to 48, 34 and 31% of their original volume, respectively [Albertyn *et al*., 1994a]. During the subsequent recovery period, the cells increase their volume again, but even for the lowest salt concentration, they do not reach their initial volume. The recovery from the osmotic shock appears to require active protein synthesis since no increase in cell volume was observed when protein synthesis was blocked with cycloheximide [Albertyn *et al*., 1994a]. The time span of the recovery period after a hyperosmotic stress has not yet been investigated and consequently there are not any references about the correlation, if any, between time span of recover, yeast tolerance levels and cytoskeleton reassembly. The genetic bases underlying the survival to an osmotic shock and the ability to adapt to and to grow at high osmolarity are not yet unraveled.

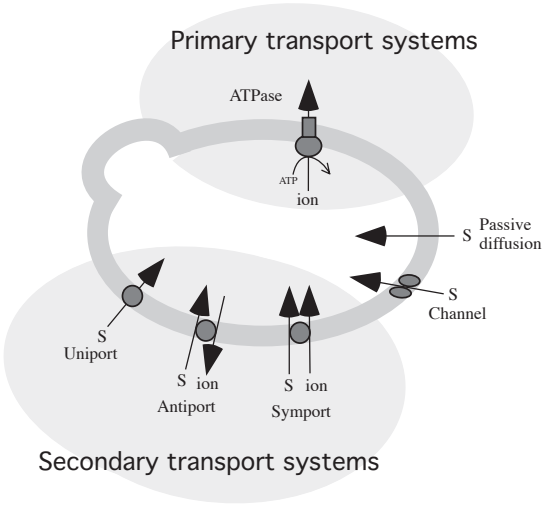
The relation between salt tolerance levels and cellular volume were, for the first time to our knowledge, established by Lages and collaborators (1999). These authors screened a group of yeasts with different tolerance levels and showed a relation between cell volume obtained upon the same degree of salt shock and the maximal intrinsic resistance to NaCl of each yeast. In spite of the limited number of yeasts tested for each class of salt tolerance, it became clear that the highest the tolerance, the less cell volume is affected by a hyper- osmotic shock [Lages *et al*., 1999].

3.1.3. *Plasma membrane transport mechanisms in yeasts*

According to their structure, plasma membrane is relatively impermeable to most hydrophilic molecules, thus there are specialized proteins or pores that mediate the selective uptake and/or secretion of solutes across these membrane. There are four basic mechanisms whereby compounds cross yeast membranes: active transport, facilitated diffusion, passive diffusion and channels. Transport systems can be classified according to several aspects. They are frequently classified according to its structure as non-mediated or protein mediated transport. Mediated transport can then be distinguished in two main types, active transport and facilitated diffusion. Non-mediated transport includes the so-called passive diffusion, which corresponds to the free passage of a substrate through the lipid bylayer. On the other hand, active transporters may be classified according to their driving force, as primary or secondary, or they can be classified according to their molecular mechanism, as uniporters, symporters, antiporters and pumps. Furthermore, they can be classified according to the electrochemical gradient as electrogenic, if they generate a gradient through the membrane (*e.g*.

ATPases), as electroforetic, if they use a pre-established gradient to operate (*e.g*. uniports, symports and antiports, in which one of the molecules is charged) and as electroneutral, if they use a pre-established gradient to operate like the electroforetic ones, but the transport of substrates does not involve a charge transference through the membrane (*e.g*. symport lactate-H^+). Figure 3.1 presents a very simple scheme of the type of transporters described for diverse substrates in yeasts.

Figure 3.1. Primary and secondary transport systems in yeasts.



Primary transport is defined as transport in which light or chemical energy is converted into electrochemical energy (*i.e.*, solute or ion concentration gradient across the plasma or organelle membrane). In the plasma membrane of *S. cerevisiae*, only ATP-driven primary transport systems have been described. For instance the hydrolysis of ATP by the major plasma membrane ATPase (Pma1p) results in the generation of an electrochemical gradient of protons (Δp-**P**roton **M**otive **F**orce-p.m.f.). This force of protons (mV) is composed of an electrical potential ($\Delta\Psi$) and a chemical gradient of protons (-ZΔpH) across the plasma membrane which is expressed as:

$$\text{p.m.f.} = \Delta p = \Delta\Psi - Z\Delta\text{pH}, \quad \textbf{[4.1]}$$

where Z = 2.3 RT/F. R is the gas constant (1.987 cal mol^{-1} K^{-1} at 1 atm of pressure and 273 K of temperature), T- the absolute temperature (ºK) and F- the Faraday constant (23.062 Kcal V^{-1} mol^{-1}). Z represents the conversion factor of pH in mV, and its value at 25ºC is 59.1. Equation 4.1 allows the estimation of the p.m.f. generated by a electrochemical proton gradient [Mitchel, 1967; deVoe and Maloney, 1980; Eddy, 1982; van den Broeck *et al*., 1982].

The p.m.f. is used widely to drive membrane associated processes such as solute transport namely active transport. In secondary transport, which includes uniport, antiport and symport, the energy for translocation of one solute is supplied by (electro)-chemical gradients of other solutes (including ions), which are often generated by primary transport systems such as the plasma

membrane ATPase. Not directly dependent of electrochemical gradient, *i.e.*, on p.m.f., are the channel mediated and facilitated diffusion transport as well as passive diffusion.

Passive or simple diffusion

Passive or simple diffusion corresponds to a non-mediated passage of substrates through the lipid bilayer [Stein, 1986]. The driving force for passive diffusion is the difference in concentration of the solute inside and outside the cell [Stein, 1986]. If a solute enters the cell by passive diffusion it is crossing the plasma membrane freely through the phospholipids bilayer. The rate of passive diffusion of solutes across the plasma membrane is governed by physical/chemical properties of the membrane such as the acyl chain length, degree of saturation of the fatty acids, membrane fluidity, and other factors [van der Rest. *et al.*, 1995]. The process of simple diffusion is not saturable with respect to the substrate and it can be experimentally distinguished from protein mediated uptake for not being equally sensitive to temperature or specific inhibitors and being stimulated by membrane fluidyzers [Stein, 1986]. A variety of sugar-alcohols such as arabinitol, erythritol, galactitol, mannitol, ribitol, sorbitol and xylitol were thought to cross the plasma membrane by passive diffusion only [Canh *et al.*, 1975]. The same was true for glycerol [Gancedo *et al.*, 1968]. However, the relatively hydrophilic nature of these solutes makes it unlikely that the rate of diffusion be, if possible, very high [Oliveira *et al.*, 2003]. Moreover, mediated transport has been already characterized for some of these compounds [Klöppel and Höfer, 1974; Loureiro-Dias, 1987; Lucas *et al.*, 1990; van Zyl *et al.*, 1990; Castro and Loureiro-Dias, 1991; Lages and Lucas, 1995, 1997]. More lipophilic compounds such as organic acids and alkanols, from which we stress ethanol, are more likely to diffuse across the membrane [Leão and van Uden, 1984; Cartwright *et al.*, 1989].

Channels

Channels or pores are structural organizations made of membrane proteins that allow the thermodinamically passive downhill flux of solutes across the plasma membrane [Stein, 1986]. They can be distinguished from permeases because their activity is less sensitive to temperature variations and the lower specificity of the transported solutes, which is determined essentially by the charge and the molecular dimension of the solute rather then their chemical structure [Stein, 1986]. Channels can function on various ways. They can react to changes in turgor or mechanic forces in the membrane or they can react, between other, to electric charges. Whatever the type of mechanism involved, they can interchange between to two possible conformations, opened and closed. In this sense, they differ from the protein-mediated transporters for not undergoing enzyme-like conformational changes as carrying the substrate/s from the outer to the inner faces of the membrane. There is, to date, three channel-type transporters represented in yeasts: (1) the channels proteins of the **M**ajor **I**ntrinsic **P**rotein family (MIP), (2) the **V**oltage-sensitive **I**on **C**hannels (VIC), and (3) the **C**h**l**oride **C**hannels (ClC) [Paulsen *et al.*, 1998].

The MIP family comprises channel proteins found in bacteria, fungi, plants, and animals whose sequences are related to the Major Intrinsic Protein of the bovine lens fibre cell membrane [André, 1995]. Proteins of the MIP family have been implicated in the diffusion of diverse substrates, including ions, water (aquaporin), glycerol and propanediol across biological membranes. Genome sequencing has revealed in *S. cerevisiae* the existence of four genes encoding proteins from MIP channels: the glycerol facilitator-like Fps1p and its close homologue Yfl054p and the water channels

Aqy1p and Aqy2p [André, 1995]. The *FPS1* gene (***F****ructose di****P****hosphatase* ***S****uppressor*) encodes for a channel which physiological function is glycerol export rather than uptake [van Aelst *et al*., 1991; Luyten *et al*., 1995]. Nevertheless, the deletion of *FPS1* reduces glycerol passive diffusion and affects membrane lipid composition, suggesting that these proteins may have additional functions in lipid metabolism and membrane organization [Sutherland *et al*., 1997]. Although *FPS1* expression is constitutive and not regulated by osmotic/salt stress, Fps1p is implicated in *S. cerevisiae* osmoregulation being responsible mainly by glycerol efflux control rather than uptake [Tamás *et al.,* 1999; Bill *et al.,* 2001]. Fps1p mediate transport of polyols, glyceraldehyde, glycine, urea and the trivalent metalloids arsenite and antimonite [Wysocki *et al*., 2001], suggesting low specificity, while only insignificant water transport has been observed through either protein. The ORF *YFL054c*, shows homology to *FPS1* and to glycerol facilitators of bacteria. Its putative function function as a glycerol transporter was observed for the first time by Oliveira and collaborators (2003).

Water transport was initially thought to occur entirely by osmosis across the lipid phase of the membrane. However, inhibition studies, activation energies and rates of transport have indicated the involvement of specific proteins and, over the past few years, water transport became well established and the aquaporins (AQPs) family was identified [Zeuthen, 2001]. *S. cerevisiae* has two genes encoding aquaporins, *AQY1* and *AQY2*, sharing more than 80% identity [Bonhivers *et al.,* 1998]. The conditions of expression and function of Aqy1p still remain to be determined. Aqy2 seems to be only expressed in rapidly growing cells and its disappearance in hypertonic medium could constitute a mechanism of protection to avoid water leakage out of the cell in hyperosmotic conditions [Meyrial *et al*., 2001].

At least two properties distinguish ion channels from the transporters that mediate solute-facilitated diffusion: (1) ion flow is extremely fast (more than 106 ions/sec), and ion channels are gated, their opening frequency being regulated by changes in membrane potential, by binding of a specific ligand, or by mechanical constraints such as membrane stretching. Essentially two types of ion channels can be found in the plasma membrane of *S. cerevisiae*: (i) voltage dependent K^+ channels and (ii) rather unspecific channels that are activated by stretching of the bilayer [André, 1995]. The predominant voltage dependent plasma membrane channel (YPK1) is an outward rectifying K^+ channel (*i.e.*, mediating K^+ efflux), which is activated by membrane depolarisation and by high cytoplasmic Ca^{2+} concentrations. The function of this channel is probably to balance charge displacements during proton-coupled substrate uptake [Gustin *et al*., 1986]. Gustin and collaborators (1988) have shown that stretching of the yeast membrane activates a less specific plasma membrane channel which conducts both cations and ions, and might play a role in osmoregulation by allowing the efflux of ions under conditions of osmotic stress. Potassium specific channels (TOK1 channels) are also represented in plasma membrane of *S. cerevisiae*, where they permeate K^+ ions. TOK1 is the first example of an outward rectifier K^+-selective ion channel and their mechanism of function may be similar to the voltage-dependent channels [Ketchum *et al*., 1995]. The predominant vacuolar channel (YVC1), which may play a role in adjusting the cytosolic Ca^{2+} concentration from the vacuolar reservoir, is a cation selective inward rectifier (*i.e.* transferring ions into the cytoplasm from the vacuole) and conducts Na^+, K^+ and Ca^{2+} [Bertl and Slayman, 1992]. Also belonging to the channel-type transporters family, the *GEF1* gene representing a chloride channel, was characterized in yeasts as participating in the high-affinity iron transport system and as having a function in cation detoxification. Thus, Gef1 seems to be required as an anion channel to provide the counterbalancing

charge that will permit cation compartmentalization into organelles or vesicles with acidic interiors [Gaxiola *et al*., 1998].

Facilitators/carrier-type transporters/permeases – Major Facilitator Superfamily

In contrast to a channel, a transporter (also called a facilitator or permease) is assumed to transfer the solute across the membrane by undergoing reversible conformational changes that expose its solute-binding site alternately on each side of the membrane. Many facilitators/transporters have been identified in yeast and where grouped in a **M**ajor **F**acilitator **S**uperfamily (MFS) which groups 17 transporter families that seemingly share common ancestral origin [André, 1995; Pao *et al*., 1998]. Some transporters, called uniporters, catalyse the transport of a charged solute down its electrochemical gradient, *i.e.*, in which the driving force is the electrochemical gradient of the transported solute. This type of transporter is dependent on temperature, saturable in relation to the substrate concentrations, and may be completely inhibited by substract structural analogues [Stein, 1986]. Electroneutral facilitated diffusion through a protein is usual in the glucose and galactose transport in *S. cerevisiae* [Bisson *et al.,* 1993; Reifenberger *et al.,* 1997; Özcan and Johnston, 1999]. Many other transporters involve the coupled movement of two (or more) solutes in the same direction -symporters, or in the opposite direction - antiporters. In yeast, these type of transporters coupled their transport with protons or other ions, which enables them to operate against the substrate chemical gradient. These symporters and antiporters are usually referred to as secondary active co-transport systems. Solutes transported by secondary transport systems can be neutral or charged and different numbers of solutes may be co- or counter-transported. As mentioned above, according to the charges transport movement, these can be classified as electroneutral, electroforetic and electrogenic.

Active transport comprises both the primary and secondary types and are used by the cell as a means to maintain high intracellular solutes concentrations, *i.e.*, they function against the natural gradient of solutes, which otherwise migth be, in some cases, lost by the cells by simple diffusion or through channels. These type of transport systems function spending energy from the cells, as ATP for primary transport or as gradients in secondary type transport, (formed using the primary transport systems). Primary active transport systems do not belong to the major facilitator superfamily and are discussed below. Many solutes like sugars, weak acids and alcohols are transported frequently in yeasts using symport with protons, in contrast with annimal cells, in which Na^+ is often used as a coupling ion [Reizer *et al*., 1994]. Antiporters catalyse the exchange of various mono- and divalent cations for protons in the plasma membrane. These systems are likely to play a role in cell volume control, regulation of cytoplasmic pH, and ion homeostasis of the cytoplasm and will further discussed in Chapter 5.

ATP-driven transport systems

The so-called primary active transport systems catalyse transmembrane solute transport in response to ATP hydrolysis, in contrast with the MFS in which the transport occurs in response to chemiosmotic ion gradients. These systems spend energy to operate against electrochemical gradients. Actually, at least two distinct families are recognized inside the ATP-driven transport systems: (i) the **A**TP-**B**inding **C**assette (ABC) superfamily, which is a family that is found ubiquitously in all classifications of living organisms and (ii) the P-type ATPases. [André, 1995]. It should be noted that

recently it was constituted a Transport Commission (TC), whose function is to classify transporters in *S. cerevisiae* genome [Paulsen *et al*., 1998; Busch and Saier, 2002]. According to this commission the ATP-driven transporters were grouped in a category in which, in turn, were recognized three families: (i) ATP-binding cassette, (ii) H^+- or Na^+ translocating (with sub-families): F-type, V-type and A-type ATPase and (iii) cation-translocating (sub-family P-type ATPase). TC has groupped and classified the transport systems according to the following four levels: (i) the first level was based on the transport mode and energy coupling mechanisms; (ii) the second level of classification was based on phylogenetic family; (iii) the third level of classification is based on phylogenetic subfamily or cluster, and (iv) the final level is based on substrate specificity [Saier, 2000; Paulsen *et al*., 1998]. The proton ATPase of the plasma membrane (*PMA1*) which supplies the driving force for a wide array of H^+-dependent co-transporters belongs to the cation-translocating P-type ATPase family [Paulsen *et al.,* 1998]. Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+, K^+), K^+ and Ca^{2+} ATPase [Serrano *et al*., 1986]. The catalytic mechanism and structural properties of P-type ATPases differ from those of the multi-subunit F-type ATPase of mitochondria and the V-type ATPase of the vacuole. Plasma membrane H^+-ATPase, one of the most abundant protein in plasma membrane [Serrano, 1991], is roughly 50% of the plasma membrane proteins and is estimated to consume 10 to 15% of the ATP produced during yeast growth [Gancedo and Serrano, 1989]. The H^+/ATP stoicheiometry of plasma membrane is 1, which differs from the 3 to 4 protons pumped per ATP by the F-type ATPase, and the pH optima are 6, 7, and 9 for plasma membrane, vacuolar and mitochondrial ATPases respectively. The differences in pH optima and sensitivity to inhibitors can be used to discriminate between the various ATPases activities. The plasma membrane H^+-ATPase is relatively sensitive to inhibitors like *ortho*-vanadate and diethylstilbestrol, whereas the vacuolar and mitochondrial ATPases are not affected by these inhibitors [van der Rest *et al*., 1995]. Addition of glucose to yeast cells causes a two- to threefold increase in plasma membrane ATPase activity being this feature widely used to search for H^+-ATPase activity in yeasts cells [Serrano, 1978; Supply *et al*., 1993].

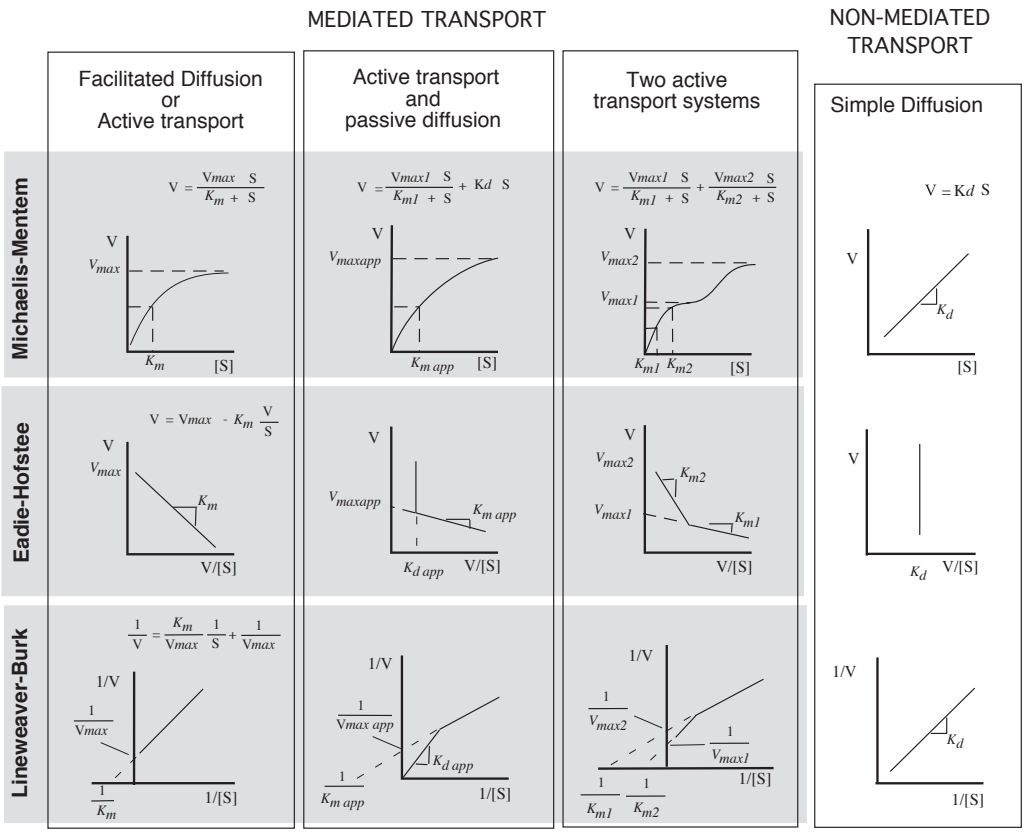
Transport kinetics

Transport systems activity can be accessed in graphical and mathematical terms. For those solutes that are transported across the plasma membrane by simple diffusion the transport along time is graphically represented by a linear equation which slope represents the diffusion constant (K_d). Uptake of solute is made proportionally to the solute concentration (Figure 3.2), but the speed of entry depends on the solubility of the substrate in the lipid bilayer. When the solutes cross the plasma membrane using a protein (permease), their uptake velocity is dependent on the capacity of transport of the protein, *i.e.*, the number of substrate molecules that bind at the same time in the protein (active center), their affinity to the substrate and the number of proteins in the membrane. Thus, carrier mediated transport is an enzyme-like reaction and carrier activity varies according to substrate concentration in a Michaels-Menten fashion, corresponding to a kinetic graphic representation as a saturation curve of velocity against substrate concentration (Figure 3.2). Michaelis-Menten enzyme kinetics defining equation have two constants: the K_m which represents the substrate affinity for a specific protein and V_{max} which gives the maximal velocity of the system, *i.e.*, the maximal number of solutes that can be transported per time, equivalent to the number of proteins in the membrane (higher V_{max} implies high expression of transport protein in the membrane). Michaelis-Menten may be algebrically transformed in two other equations widely used in the determination of kinetic parameters

which are the Lineweaver-Burk and Eadie-Hofstee equations (Figure 3.2). Solutes can be simultaneously transported by two or more different type of transport systems. In these cases equations representing bulk transport must have more than one branch. Figure 3.2 presents not only the graphical but also the respective equation of the most usual situations in which the solute is transported by one or two types of transport systems.

When two systems operate at the same time, the mathematical equations do not represent accurately each of the systems, since the systems overlap each other (Figure 3.2). Results must thus be analyzed by appropriate software which iterate the contribution of each type of uptake to global experimentally determined apparent transport kinetics parameters. GraphPad PRISM is a software currently used to analyze the results and allows the choice of the best equation fitting the experimental results by using non-linear regression analysis [Fuhrmann and Volker, 1992].

Figure 3.2. Kinetics of transport systems. Mathematical and graphic representation.



V- initial velocity of solute transference; **S** - solute concentration; ***Vmax*** - transport system maximal velocity; ***Km*** - Michaelis-Menten constant ***Kd*** - diffusion constant

In order to measure uptake of solutes and to verify the possible co- or counter-transport with protons we use two parallel approaches: to measure uptake using radiolabelled substrates and to

measure extracellular alkalinization or acidification upon the addition of the same solutes to an unbuffered cell suspension, according to methodologies widely used in transport measurements [Lucas and van Uden, 1986; Loureiro-Dias, 1987, 1988; Lucas *et al*., 1990; Cássio, 1994; Casal, 1995; Lages and Lucas, 1994, 1995, 1997; Gerós, 2000]. Furthermore, it is also common to study the effect of several drugs, some of them known to act as ionophores, such as carbonylcyanide *n*-chlorophenylhydrazone (CCCP), tetraphenylphosphonium (TPP), 2,4-dinitrophenol (DNP), nigericin, and valinomycin, and other as inhibitors of the H^+-ATPase plasma membrane such as *ortho*-vanadate, diethylstilboestrol (DESB) and N,N´-dicyclohexylcarbodiimide (DCCD) (Table 3.1) to characterize energetically the transport systems functioning. Ionophores are molecules that present an external hydrophobic region which can bind selectively protons or other ions forming specific lipossoluble complexes [Pressmann, 1976]. These can cross the lipid bilayer in their charged chemical form contributing to dissipate their transmembrane gradient and thus affecting p.m.f. [Pressmann, 1976]. The effects of all these drugs are tentatively summarized in Table 3.1. Their effective inhibiting concentration depends on the type of organism in which they are used as well as experimental conditions.

Table 3.1. Drugs widely used in transport assays and their mode of action [Serrano, 1980; Goffeau and Slayman, 1981; Harold, 1986; Dawson *et al*., 1986].

Drug name	Mode of action
CCCP (Carbonylcyanide *n*-chlorophenylhydrazone)	Protonophore that permeabilizes the membrane for protons or ions by promoting the symport of H^+ and anions in one direction and the anion in the opposite direction. Effective uncoupler of oxidative phosphorylation in mitochondria.
DNP (2,4-Dinitrophenol)	Uncouples oxidative phosphorylation by mediating proton conductance across inner mitochondrial membrane; stimulates mitochondrial ATPase.
TPP (Tetraphenylphosphonium)	This cation is able to distribute uniformly in the two sides of the membrane, thereby destroying the electrical gradient naturally established by the membrane.
Nigericin	Carboxylic ionophore; promotes selectively the electroneutral antiport of protons and the ions K^+, Rb^+, Na^+ and Li^+.
Valinomycin	Neutral ionophore; promotes selectively the electrogenic uniport of the ions K^+, Rb^+, Na^+, Li^+ and H^+.
DCCD (N,N´-dicyclohexylcarbodiimide)	Non-specific inhibitor of the plasma membrane ATPase.
Sodium *Ortho*-vanadate	Specific inhibitor of the plasma membrane ATPase.
DESB (Diethylstilbestrol)	Uncouples oxidative phosphorylation; inhibits Na-K-ATPase; specific inhibitor of the plasma membrane ATPase.

Ethanol has been used for a long time as an uncoupler of the plasmatic membrane increasing proton influx [Leão and van Uden, 1984; Cartwright *et al*., 1986]. This increase dissipates the proton motive force [Leão and van Uden, 1984; Cartwright *et al*., 1986], and thus interferes specially with transport systems which are dependent on the electrochemical gradient of protons. From a kinetic point of view, this is translated by a non-competitive exponential inhibition of substrates mediated transport by ethanol [van

Uden, 1985, 1989], which is expressed by the following equation:

$$V_{max} = V_{max}^{\ 0}\, e^{[-Ki\,(C\text{-}Cmin)]}\,[S/(K_m+S)] \qquad \textbf{[3.2]}$$

V_{max} and $V_{max}^{\ o}$ represent the maximal velocities in the presence and in the absence of ethanol at the C concentration, respectively. K_i represents the inhibition exponential constant and C_{min} the minimal solute concentration below which is not possible to detect any inhibition effect. Furthermore, the characterization of transport systems also involves the determination of the variation of V_{max} according to external pH as well as transport specificity and the estimation of the affinity constants for different compounds able to permeate through the same transporter.

Although a considerable amount of data is available on the transport processes in the plasma membrane, the translocation mechanisms and the factors that control the rate of transport are still poorly understood. The development of protein engineering methodologies, spectrometry and other methodologies able to unveil protein structures can contribute to a deeper understanding of transporters/enzymes chemical/molecular function mechanisms. Furthermore, an increase in knowledge on the membrane structure and the role of the localized biophysical/biochemical environments around intrinsic proteins would be crucial in order to unveil translocation catalysis operation, protein conformational changes and so on [van der Rest *et al*., 1995].

3.1.3.1. *Glucose transport*

The first step in sugar metabolism is the transport of the sugar across the plasma membrane. Yeasts possesse a large diversity of hexose transport systems, usually grouped in two categories: (i) active transport, more frequently as a symporter with protons as it occurs for glucose in *S. pombe* [Höfer and Nassar, 1987], *C. utilis* [Peinado *et al*, 1989; van den Broek *et al*., 1997], *Candida intermedia* [Loureiro-dias, 1987], *Candida wickerhamii* [Spencer-Martins and van Uden, 1985], *Candida shehatae* [Lucas and van Uden, 1986] and for fructose in *Saccharomyces carlsbergensis* [Gonçalves *et al*., 2000], *Saccharomyces bayanus* and *Saccharomyces pastorianus* [de Sousa *et al*., 1995]; and (ii) facilitated diffusion like in *S. cerevisiae* hexose transport family [Bisson *et al*., 1993; Reifenberger *et al*., 1997; Özcan and Johnston, 1999].

In the yeast genome 20 homologues of hexose transporters genes have been identified [André, 1995; Boles and Hollenberg, 1997; Paulsen *et al*., 1998; Özcan and Johnston, 1999]. The family of homologues hexose transporter genes includes *HXT1-HXT17* (**H**e**X**ose **T**ransport), *GAL2* (**GAL**actose), *SNF3* (**S**ucrose **N**on **F**ermenting) and *RGT2* (**R**estores **G**lucose **T**ransport), and is part of the major facilitator superfamily of transporters [Pao *et al*., 1998]. *HXT1-HXT4* and *HXT6-HXT7* encode the relevant hexose transporters for growth on glucose. Kinetic experiments on the individual transporters showed that *HXT1* and *HXT3* encode transporters with low affinity for glucose (Km = 50-100 mM), *HXT4* encodes a transporter with moderate affinity for glucose (Km ≈ 10 mM), and *HXT6* and *HXT7* encode high affinity (Km = 1-2 mM) hexose transporters [Reifenberger *et al*., 1997]. In addition to glucose, the hexose transporters show also affinity for fructose and mannose [Lagunas, 1993; Reifenberger *et al*., 1997]. During exponential growth on excess glucose, glucose transport exhibits low affinity kinetics, but as glucose gets depleted the affinity for glucose increases. In aerobic glucose-limited chemostat cultures of *S. cerevisiae*, the affinity of the hexose transport was correlated to the amount of extracellular hexoses [Diderich *et al*., 1999a].

SNF3 and *RGT2* are highly homologous to the *HXT*-genes, however, *SNF3* expression was shown to be much weaker than that of *HXT*-genes and not sufficient to confer uptake of glucose. A

role in metabolism, namely as sensors, rather than in metabolism became apparent. It was found that *SNF3* is required for the expression of *HXT*-genes at low levels of glucose, while *RGT2* encodes a protein that is involved in the expression of *HXT*-genes at high levels of glucose [Özcan *et al*., 1996]. *GAL2* encodes for the galactose permease, which mediates transport of galactose and that of glucose with high affinity [Reifenberger *et al*., 1997].

Besides *S. cerevisiae*, glucose uptake has been stated also in plenty other yeasts although not so deeply as in *S. cerevisiae* [Loureiro-Dias, 1988; Özcan and Johnston, 1999]. Glucose transport seems to occur in *K. lactis* by facilitated diffusion [Wésolowski-Louvel *et al*., 1992]. Two genes were identified that encode for transporters with different affinities to glucose. *HGT1* encodes for a constitutive transporter exhibiting high affinity (Km ≈ 1mM), while *RAG1* encodes for an inducible transporter of glucose and fructose with low affinity (Km ≈ 20-50 mM) [Wésolowski-Louvel *et al*., 1992; Billard *et al*., 1996]. *RAG1* expression is quite low in gluconeogenic conditions, being induced in media containing sugars such as glucose, fructose, mannose, sucrose, raffinose and galactose [Chen *et al*., 1992]. *C. utilis* is able to transport glucose through a symport with protons when cultured in the presence of low glucose concentrations and by facilitated diffusion, similarly to *S. cerevisiae*, for high glucose concentrations in the media [Peinado *et al*., 1989; van den Broek *et al*., 1997]. Weierstall and collaborators (1999) isolated three genes encoding glucose transporters in *P. stipitis*. *SUT1* is one of the genes and is induced by glucose, while *SUT2* and *SUT3* are constitutively expressed but only in aerobic conditions. These transport proteins were also able to transport xylose and other monosaccharides, but with considerably lower affinity [Weierstall *et al*., 1999]. In the case of *Y. lipolytica*, glucose kinetics, independently of glucose concentration, always exhibits a two component system, one with an affinity of 3 mM and other with 10 mM [Does and Bisson, 1989]. In the fission yeast *S. pombe* a family of six hexose transporters *GHT1-GHT6* was also identified [Höfer and Nassar, 1987; Mehta *et al*., 1998; Heiland *et al*., 2000]. *GHT1*, *GHT2* and *GHT5* exhibit higher activity for glucose than for fructose [Heiland *et al*., 2000]. More recently, it was identified in *C. albicans* a family of 21 hexose transporters, Ca*HGT1* and *HGT1-HGT20* [Varma *et al*., 2000; Fann *et al*., 2002]. It is likely that most of the yeasts possesses several sugar transporters, enabling survival in a variety of environments from wineries to nature [Luyten *et al*., 2002]. An example is the case reported for the kinetics of glucose uptake in *Dekkera anomala*, a very slow growing but serious wine spoilage yeast with high impact in wine making industry [Gerós, 1999]. The high affinity glucose transport system identified in *D. anomala* in conditions with low glucose concentrations, may justify the ability of this yeast to grow and spoil the wine in the last stage of wine making, when glucose concentration is quite low [Gerós, 1999].

3.1.3.2. *Polyol transport*

Acyclic polyols such as erythritol, xylitol, ribitol, D-arabinitol, mannitol, sorbitol and galactitol, are a group of linear sugar derivatives that can serve as reserve substances and osmotic regulators in a number of microorganisms [Canh *et al.*, 1975]. Their metabolism has been studied thoroughly in different yeasts species [Barnett, 1968; Jennings, 1984], but the same did not happen to their transport across the plasma membrane. *S. cerevisiae* does not metabolise significantly acyclic polyols, but it was reported that they are transported through the plasma membrane by a non saturable process [Canh *et al*., 1975]. In the case of *R. glutinis* [Klöppel and Höfer, 1974] and *T. candida* [Haskovec and Kotyk, 1973], they showed to actively transport polyols against a concentration gradient. Two transport systems were reported to exist in *R. glutinis*: one low affinity constitutive system and a high affinity inducible

system, being this last specific for pentitols [Klöppel and Höfer, 1976]. Polyol transport as also studied in *C. intermedia* [Loureiro-Dias, 1987]. This yeast showed to be able to transport sorbitol, xylitol, D-arabinitol and mannitol through a specific H^+-polyol symport system inducible by growth on sorbitol [Loureiro-Dias, 1987].

Glycerol

Glycerol is the osmolyte most used by yeasts, furthermore many yeast are able to use it as carbon and energy source (Chapter2). Glycerol was thought to permeate freely across the yeast plasma membrane [Gancedo *et al.,* 1968]. Actually, it was referred that the difference between the *C. utilis* and *S. cerevisiae* efficiency for glycerol utilization was due to the different degrees of membrane permeability to this compound of these two yeasts, high and low respectively [Gancedo *et al.,* 1968]. Other authors considered glycerol ability to permeate the yeast membrane a fact and it was even used as a measure of membrane leakiness state [Pesti and Novák, 1984; Ushio *et al*., 1991]. Recently it has been shown that most of the so-called glycerol passive diffusion is actually done through Fps1p channel once opened [Oliveira *et al.,* 2003]. Accordingly, most of the first-order kinetics found for glycerol uptake is absent from *fps1* mutants [Oliveira *et al.,* 2003].

Glycerol transport was described for the first time in eukaryotes by Castro and Loureiro-Dias (1991) in the filamentous fungus *Fusarium oxisporum* var *lini.* In this fungus glycerol uses a facilitated diffusion system which is shared by 1,2-propanediol, partially constitutive, and subjected to repression and inactivation by glucose. Adler and collaborators (1985) had already suggested the presence of an active transport for glycerol in the halotolerant yeast *D. hansenii*, and in 1990, Lucas and collaborators, characterized this transport suggesting a model of a co-transport of glycerol with either Na^+ or K^+ ions. van Zyl and collaborators (1991) also postulated the existence of mediated transport for glycerol in *Z. rouxii* with identical characteristics. Later, Lages and collaborators (1995) characterized completely the transport system of glycerol in the halotolerant yeast *P. sorbitophila*. This yeast was shown to possess an active transport system for glycerol of the H^+/symport type constitutive and highly specific. Glycerol was able to permeate through *P. sorbitophila* cells by this active transport system and simultaneously by, apparently, *passive diffusion.* In the case of *S. cerevisiae*, glycerol transport has been a matter of intense and controverse studies. Results point to glycerol being permeated in this yeast by a constitutive proteic-channel encoded by the *FPS1* gene mentioned above, implicated in *S. cerevisiae* osmoregulation, namely in glycerol efflux regulation [Tamás *et al*., 1999; Bill *et al*., 2001]. Besides, it was also reported by Lages and Lucas (1997) an inducible H^+-symporter for glycerol in *S. cerevisiae*. This was shown to be under glucose repression, induced by growth on non-fermentable carbon sources and unrelated with salt/osmotic stress [Lages and Lucas, 1997]. Glycerol transport knowledge in yeasts was greatly improved with the study made by Lages and collaborators (1999). These authors clearly showed that active glycerol uptake constitutive presence is related to high salt-stress resistance and thus that it may be advantageous for survival under salt stress.

Mannitol

Mannitol is the most abundant sugar alcohol found in nature, occurring in bacteria, fungi, lichens, and many species of vascular plants [Williamson *et al*., 1995]. Furthermore, as said in chapter 2, it is able to perform several important metabolic functions. However, mannitol transport

characterization is scarce. In spite of being unable to metabolize mannitol, *S. cerevisiae* was used to study mannitol transport. It was found to cross the membrane according to a saturable uptake which was not further characterized [Maxwell and Spoerl, 1971]. Later, it was otherwise shown to be taken according to a first-order kinetics indicative of passive diffusion [Canh *et al.*, 1975]. Mannitol uptake was also studied in the opportunistic yeast *C. albicans* [Niimi *et al.,* 1986] and in more detail in the halotolerant yeast *P. sorbitophila* [Lages, 2000]. In this last case it was found to be transported through a proton symporter shared by erythritol, D-arabinitol, xylitol and sorbitol. This study presented a rather unusual event, corresponding to the fact that proton movements associated with polyols uptake only being detected in cells shocked in 1M NaCl.

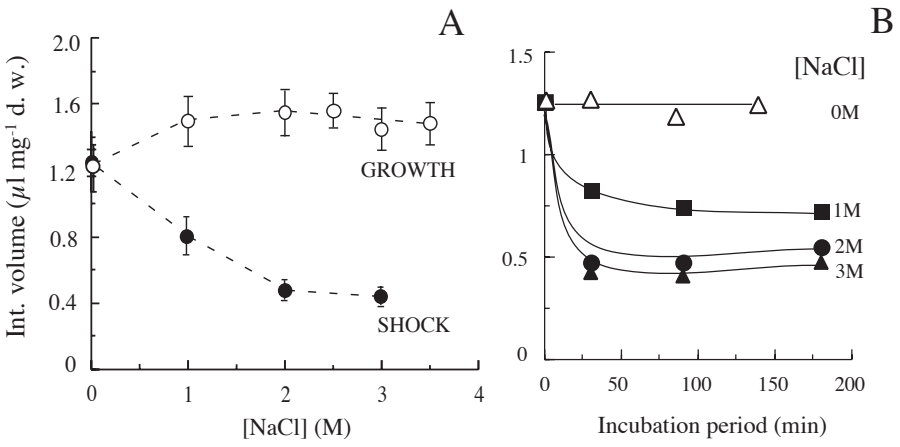
As said before in Chapter 2, mannitol is present in more than 100 species of higher plants, where it can be a significant portion of the soluble carbohydrate [Williamson *et al*., 1995]. Thus identification and characterization of mannitol transporters is specially important in plants because mannitol is both a primary photosynthetic product, a compatible solute and antioxidant implicated in resistance to biotic and abiotic stress [Noiraud *et al*., 2001]. Celery is a mannitol-synthesizing plant that has been used as a model to study mannitol metabolism in relation to various stresses [Stoop *et al.,* 1996]. Mannitol transport was characterized in the celery phloem and found to be co-transported with protons and regulated by glucose [Noiraud *et al*., 2001].

3.2. Results and discussion

3.2.1. C. halophila *cell volume under salt stress*

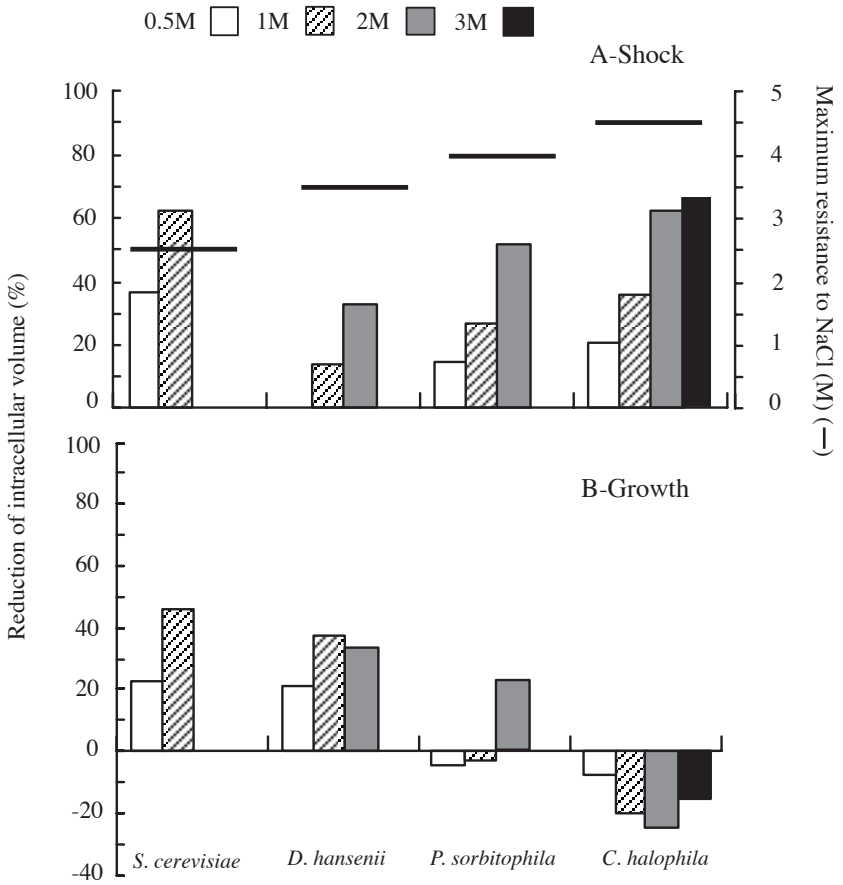
Candida halophila cell volume variations according to salt shock were determined, and these were compared to cell volumes measured in cells cultured under identical degrees of salt stress (Figure 3.3).

Figure 3.3. **A**: Intracellular volume of *C. halophila* cultivated or shocked for 30 min incubation time in MM with 2% (w/v) glucose, at 30ºC, in the presence of different NaCl concentrations (average values of at least six replicates of three independent batches). **B**: Variation of intracellular volume by salt shock along incubation period in identical conditions as in A.



C. halophila cells, as expected, reduced their cellular volume, in an inverse relation with salt concentration. Instead, when growing actively in the presence of salt, not only did its volume decrease, but it even slightly increased (about 20%) with increasing salt concentrations. Cell volume determinations under salt shock were made at different assay incubation times (Figure 3.3-B). Cell volume value does not show any variation for periods of incubation higher than 30 minutes. The same type of results has been observed in *P. sorbitophila* [Lages and Lucas, 1995], but only for concentrations up to 2M, as can be seen in Figure 3.4. We have searched literature and compared the response to NaCl, in terms of volume variations, of several yeasts (Figure 3.4).

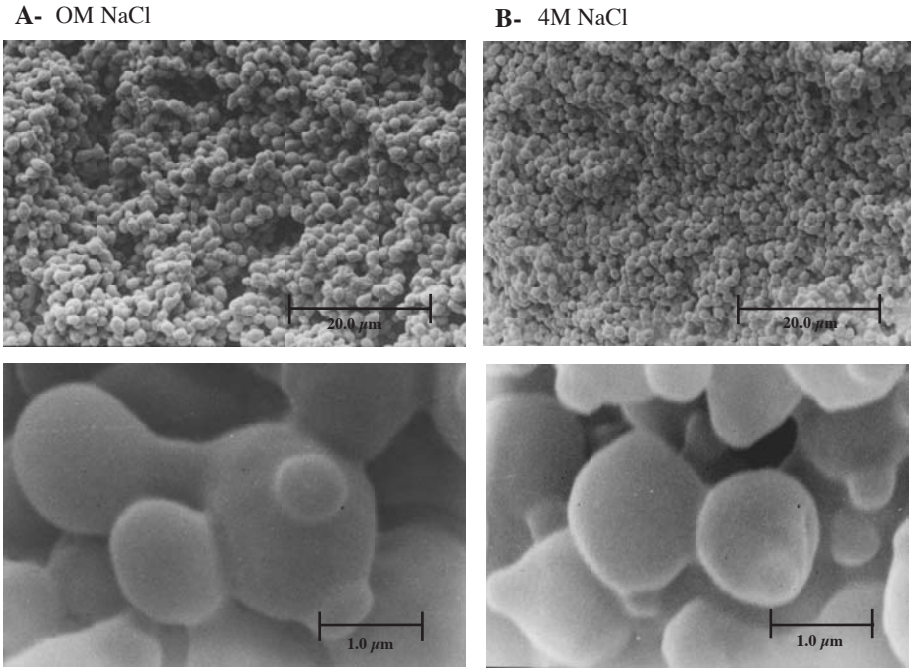
Figure 3.4. Percentage of cell volume reduction under salt shock (**A**) and after adaptation to salt stress during growth (**B**), using values obtained during the work with *C. halophila* and data from other authors, for yeasts representing different degrees of salt tolerance (horizontal lines). Species were ordered according to their maximum tolerance to NaCl. Shock: *S. cerevisiae* and *P. sorbitophila* [Lages, F. unpublished results; Lages and Lucas, 1995], *D. hansenii* [Norkrans; 1969; Norkrans and Kilin, 1969]; Growth: *S. cerevisiae* and *P. sorbitophila* [Lages, F. unpublished results], *D. hansenii* [Prista *et al*. 1997]. Initial values of intracellular volume of cells grown without salt in µl mg^{-1} dry weight were *C. halophila*- 1.25; *S. cerevisiae*-1.9; *P. sorbitophila*-3.2, *D. hansenii*-1.5



The Figure separates the responses obtained by shocking the cells (Figure 3.4-A) from the results obtained in cells growing exponentially in salt media (Figure 3.4-A). *S. cerevisiae*, as a non tolerant yeast showed the highest values for reduction of cellular volume both when shocked or when growing in the presence of salt, and thus the pattern of variation is very different from the other represented yeasts. When comparing results of *D. hansenii*, *P. sorbitophila* and *C. halophila* we realize that some relation could be established between the reduction in intracellular volume and the maximum intrinsic tolerance of each strain (Figure 3.4). This correlation had already been suggested before by Lages and collaborators (1999). The more halotolerant yeasts such *C. halophila* and *P. sorbitophila* are the ones that suffer the highest decrease in intracellular volume after shock, but are also the ones that are able to recover more efficiently during growth and even increase their volume as compared with non-stress conditions (Figure 3.4). These observations point clearly for a relation between the maximal resistance of yeasts and the percentage of reduction of cellular volume, which may suggest that yeasts surviving extreme salt concentrations have evolved mechanisms that allow them to regain their normal cellular status as in the case of *C. halophila*. Together, the results obtained before in *S. cerevisiae* and those presented in Figure 3.4, the results from *P. sorbitophila* and those of *C. halophila* strongly suggest that these yeasts have different abilities when facing salt stress and *C. halophila* seems to be the only one that is able to fully recover volume in any of the salt stress conditions assayed. Alterations in cellular volume after osmotic shock could be due to differences in the cell wall rigidity of the yeasts, *i.e.* the less osmotolerant yeasts having less rigid cell walls than the more osmotolerant yeasts, as suggested by Blomberg and Adler (1992).

C. halophila volume variation in the presence of salt can be observed by micrographs obtained by **S**canning **E**lectron **M**icroscopy (SEM) (Figure 3.5).

Figure 3.5. SEM observations of *C. halophila* cells grown in MM with 2% (w/v) of glucose as carbon source in the absence of salt (**A**) and cells grown in the presence of 4M of NaCl (**B**).



As can be seen in Figure 3.5, and considering the extent of stress applied (4M), the morphology, the volume and the shape of *C. halophila* cells do not appear to vary significantly from cells cultivated without salt (A) to cells which were grown in salt rich media containing 4M of NaCl (B). Hamada and collaborators (1984) observed also cells of *S. rouxii* grown without salt and in the presence of 2.6 M of NaCl by SEM, and did not find differences in volume, although the cells grown in a medium containing salt were spherical, whereas those grown in the presence of salt were spheroidal. This result suggested, according to those authors, that the cell wall structure might undergo some changes at higher concentrations of salt. *Y. lipolytica* cells incubated in the presence of 1.5 M of NaCl were also observed by SEM and a reduction in volume accompanied by an alteration in the cell shape was verified [Andreishcheva *et al*., 1999]. On the contrary to what happened to *Z. rouxii* cells, *Y. lipolytica* cells switched from a speroidal shape to a spheric shape when cultivated without and with salt, respectively [Andreishcheva *et al*., 1999]. Results on microscopic observations seem to confirm those obtained for intracellular volume determination, since more halotolerant yeasts such as *C. halophila* and *Z. rouxii* did not exhibited a reduction in cellular volume in contrast to the less tolerant yeast *Y. lipolytica*.

3.2.2. *Transport systems in C. halophila*

3.2.2.1. *Glucose uptake study*

Glucose transport characterization

C. halophila cells grown in MM using 2% (w/v) of different carbon sources such as glucose, glycerol, mannitol and ethanol were assayed for glucose transport. Glucose uptake kinetics of *C. halophila* cells is presented in Figure 3.6 and results exhibit Michaelis-Menten kinetics, which suggests that a protein is involved in glucose transport. Glucose transport maximal velocity is enhanced in all carbon sources tested when compared with glucose grown cells (2% w/v), being this accompanied by an increase in the affinity for the transporter protein (Figure 3.6; Table 3.2). Furthermore, the transport of glucose in media containing 0.1% (w/v) of glucose is highly increased when compared with those of cells growing in 2% of glucose.

When yeasts possess several transporters for uptake of sugars, the sugar concentration in the media is usually the key in transport systems regulation [Lagunas, 1993; Bisson *et al*., 1993; Diderich *et al*., 1999]. Generally, high affinity transporters are repressed by high sugar concentrations in the media, being this effect diminished as soon the sugar concentration is reduced [Diderich *et al*., 1999]. This type of regulation is well documented for glucose transport system in *S. cerevisiae* and *D. anomala* [Lagunas, 1993; Bisson *et al*., 1993; Gerós, 1999; Diderich *et al*., 1999b]. Furthermore, the increase observed in glucose transport V_{max} and in K_m in cells growing in carbon sources such as glycerol, mannitol and ethanol is also reported in literature and seems to be the result of the relief of the repression caused by the sugar concentration. Transport enhancement in *C. halophila* was more pronounced in mannitol growing cells, which could suggest the existence of several states of repression according to the carbon source. Whether we are in the presence of one transport system regulated by the repression state of the cell or by two transport systems having different transport kinetics, can not be elucidated by these kinetic results and further assays have to be performed. Transport involving proteins may be of facilitate or active type. In order to clarify what type of transport *C. halophila* cells are using in glucose uptake, we performed additional assays.

Figure 3.6. Michaelis-Menten (**A**) and Eadie-Hofstee (**B**) plots of initial uptake rates at pH 5.0 of D-[^{14}C]-glucose by cells of *C. halophila* grown in MM with different carbon sources: Glucose 2% (w/v) (●), 0.1% (w/v) (○); **B**-Glycerol 2% (w/v) (■); **C**-Mannitol 2% (w/v) (▲); **D**-Ethanol 2% (w/v) (□).

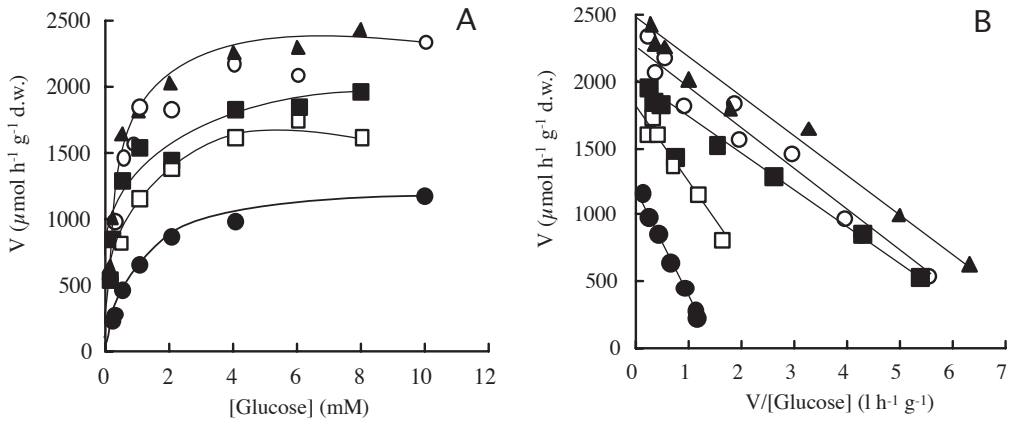


Table 3.2- Kinetic parameters of the initial uptakes rates of labelled glucose in cells of *C. halophila* grown in MM supplemented with 2% (w/v) of different carbon sources. Data obtained by non-linear regression analysis using GraphPad Prism software.

Carbon source	**Km** (mM)	**Vmax** (μmol h^{-1} g^{-1} d.w.)
Glucose 2% (w/v)	1.04 ± 0.47	1270 ± 120
Glucose 1% (w/v)	0.33 ± 0.05	2323 ± 100
Glycerol	0.28 ± 0.03	2030 ± 800
Mannitol	0.51 ± 0.33	2420 ± 210
Ethanol	0.42 ± 0.30	1800 ± 830

We looked for external pH changes following glucose pulses in *C. halophila* cells, and observed that glucose addition elicited an intensive acidification of the extracellular pH, which is known to be a response related to the activity of the plasma membrane H^+- ATPase [Serrano, 1983].Curiously it was reported that the H^+-ATPase of *C. versatilis* (the specie into which *C. halophila* was reclassified) was insensible to a glucose pulse, but stimulated by salt [Watanabe *et al*., 1993a]. Glucose transport was assayed in the presence of several inhibitors according to the Table 3.3, in order to verify if the glucose transporter albeit not presenting external alkalinization upon glucose addition might still depend on p.m.f..

Table 3.3. Effect of different drugs such as uncouplers and specific H^+-ATPase inhibitors on radiolabelled glucose uptake expressed in percentage of reduction of *Vmax*. Cells grown in MM at pH 5.5 with glucose 2% (w/v) as carbon source. Values are the mean of at least three independent assays.

Drug	Drug concentration	% of reduction of glucose transport *Vmax* *
CCCP	40 μM	50 ± 4
Nigericin	0.1 μM	72 ± 6
DCCD	2 mM	63 ± 2
DESB	5 mM	71 ± 2
Sodium *ortho*-vanadate	80 μM	16 ± 2
TPP	16 mM	63 ± 4

*100%-*Vmax* 1266 μmol h^{-1} g^{-1} dry weight

CCCP is a strong uncoupler of the p.m.f., *i.e.*, eliminates the transmembrane proton gradient, and for active uptake systems, which depend and are energized by proton gradients, 40 μM is enough to reduce almost 100% the maximal velocity. In the case of glucose uptake this reduction accounts to only 50%, for which we have no explanation, since this inhibition percentage does not distinguish clearly enough whether an active transport system is involved. CCCP results, by themselves do not allow to withdraw firm conclusions. For instance, Nobre and collaborators (1999) used 50 μM of CCCP over a xylose-proton symporter and obtained only 67% of inhibition, but over glucose-proton symporter, a 82% of inhibition was observed. These results, in the absence of proton signals will not allow definitive conclusions but imply extreme care in their interpretation. Protonophores such CCCP and DNP provoke a massive influx of protons into the cell in exchange of K^+ ions. According to Serrano (1991) the inhibition obtained in these cases may be, in part, caused by intracellular pH acidification.

Plasma membrane H^+-ATPase activity measured in membrane fractions is relatively sensitive to *ortho*-vanadate and DESB at concentrations of 100 μM, being reduced to 50 and 16 % activity, respectively in *S. cerevisiae* [van der Rest *et al.*, 1995]. Since we used, at least for DESB, higher concentrations of this inhibitor and obtained also a high percentage of inhibition of the transport it is unclear whether the concentration of the inhibitor is acting at the transporter level or is producing other effects at the metabolism level. Sodium *ortho*-vanadate has been generally claimed to be a specific inhibitor of the H^+-ATPase (Table 3.1) however, *in vivo* and *in vitro* studies have shown an inhibitory effect of vanadate not only in Na^+/K^+ and P-type ATPases [Cantley *et al.*, 1978; Karlish *et al.*, 1979; Wach and Graber, 1991] but also in RNases [Linquist *et al.*, 1973], protein-acid- and alkaline phosphatases [Foulkes *et al.*, 1983], phosphofructokinase and adenylate kinase [Chasteen, 1984]. In fact, sodium *ortho*-vanadate did not affect the glucose uptake significantly in *C. halophila* cells, and because its action in cells may be widespread, we cannot conclude about the implication of ATPase on glucose transport in *C. halophila* cells through the use of this compound. DCCD is a non-specific inhibitor and at concentrations of 1 μM does not affect the H^+-ATPase activity, instead it interferes with mitochondrial ATPase. Actually, Goffeau and Slayman (1981) advised that although vanadate and DESB discriminate between plasma membrane, mitochondrial and vacuolar ATPases, in membrane fractions, being potent inhibitors of the plasma membrane, a special care should be taken when using them in whole-cell studies. In fact DESB was shown to disrupt artificial lipid bilayers at high

concentrations [Weissman *et al.*, 1976]. DCCD induced a reduction in glucose uptake similar to the one of DESB, and thus it might be a consequence of other effects than at the plasma membrane H^+-ATPase activity. Surprinsingly, nigericin, which catalizes the antiport of protons with, namely, potassium ions and dissipates the pH gradient (also acting as an uncoupler), when used at such low concentrations as 0.1 μM produced a high reduction in glucose uptake. According to Kovác and collaborators (1982) valinomycin and nigericin act preferentially on mitochondria and do not affect fluxes of ions across the plasma membrane of the yeast *S. cerevisiae* or their permeability. Thus, DESB and nigericin results in *C. halophila* might indicate that changes in mitochondria ion fluxes are important in glucose uptake. TPP is a lipophilic cation and has been also used for membrane potential measurement. Nevertheless, its depolarising capacity at high concentrations has created some restrictions in its actual use in the determination of the yeast electric potential ($\Delta\Psi$) [de la Peña *et al*., 1982; Prasad and Höfer,1986; Bakker *et al*.,1986]. The percentage of inhibition of TPP on glucose uptake was considerable, although we should look this result carefully. In fact, we used a concentration that might already be considered too high, but for lower concentrations and longer incubation times we did not observe any inhibition. Therefore the results obtained, once more, may represent a moderate dependence on electric potential but may also be a consequence of effects at several cellular levels in a similar way as postulated for DESB and nigericin. It is of general consensus that the use of drugs in transport characterization and their interpretation must be carefully conducted. Together, the results suggest that movement of protons through the plasma membrane do not seem to be implicated with glucose uptake, confirming the possibility of glucose being transported through a facilitated difusion type system.

It is current practice to use the inhibitory effect of ethanol in substrate uptake to distinguish between non-mediated and mediated substrate entry. As membrane fluidizer, it destabilizes intrinsic proteins affecting their catalytic activity. Accordingly, substrate passive diffusion passage through the membrane, is stimulated. As uncoupler, ethanol inhibits active transport. This way, the inhibitory effect of ethanol is generally stronger on active transporters which depend on p.m.f, than on equilibrating-type transporters such as facilitated diffusion.

The effect of ethanol over glucose uptake was, as expected [van Uden, 1985], a non-competitive inhibition type (not shown). The variation of transport *Vmax* inhibition percentage with increasing ethanol concentrations followed the exponential curve in Figure 3.7.

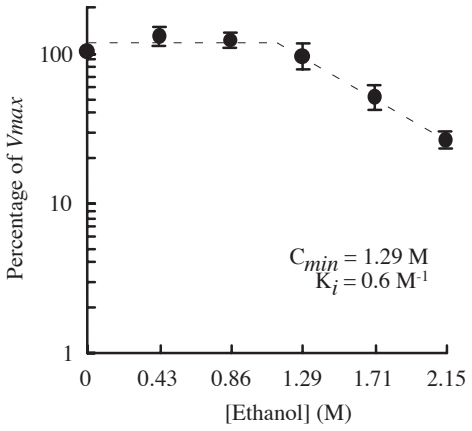


Figure 3.7. Ethanol effect over glucose transport in *C. halophila* cells grown in MM at 30ºC with 2% (w/v) glucose as carbon source and assayed in 100 mM Tris-citrate buffer, pH 5.0, having different concentrations of ethanol. Cells were incubated 2 minutes in buffer containing ethanol and then assayed for glucose transport. Glucose uptake Vmax variation in percentage is represented as a function of ethanol concentration.

Exponential inhibition constant (K_i) and the minimum inhibitory ethanol concentration (C_{min}) were determined (Figure 3.7). Experiments were performed to allow the determination of the ethanol exponential inhibition constant (K_i) and the minimum inhibitory concentration (C_{min}) (Figure 3.7). The comparison of inhibition parameters of ethanol over glucose uptake in *C. halophila* (Figure 3.7) and other yeasts (Table 3.4.) reveals that the glucose transport in *C. halophila* is less sensitive to ethanol effects than *S. cerevisiae* or *C. shehatae*, since it presents a rather high C_{min}. On the other hand, the K_i value resembles the ones obtained in other yeasts for facilitated diffusion systems. Actually, *C. halophila* constants are very close to the ones obtained for facilitated diffusion in *D. anomala* (Table 3.4).

Table 3.4. Some examples of ethanol inhibition parameters on glucose uptake in yeasts and their comparison with those of *C. halophila*. K_i – exponential inhibition constant; C_{min} – minimum inhibitory concentration.

Yeast	K_i (M^{-1})	C_{min} (M)	Reference
C. halophila			
facilitated difusion	0.6	1.29	present work
S. cerevisiae			
facilitated difusion	0.62	0.33	van Uden, 1985
D. anomala			
facilitated difusion	0.5	1.60	Gerós, 1999
C. shehatae			
facilitated diffusion	0.79-0.99	0.46-0.68	Lucas, 1987
H^+-glucose symporter	1.47	0.39	
D. hansenii			
facilitated diffusion	0.60	≈ 0.0	Nobre *et al*., 1999
H^+-glucose symporter	0.98	0.86	

According to Gerós (1999) the low sensitivity of *D. anomala*, may explain the ability of this yeast to spoil wine in the last stage of wine making, where high concentrations of ethanol and lower glucose concentrations prevail. *C. halophila* has been isolated from soy mash which is used in soy sauce production. Soy sauce composition varies during a six months fermentation process in which the microbial action is carefully controlled. The flavour and colour of soy sauce is the result of the formation of several compounds such as amino acids, sugars, alcohol and various acids. Thus, it is likely that *C. halophila* might be naturally less sensitive to compounds such as ethanol. If the glucose transport system in *C. halophila* is of electroneutral uniport type, the inhibitory effects of ethanol may be explained exclusively by interference with the porter protein, either directly or by changing its lipid environment in the plasma membrane. In the case of an electrogenic uniport or proton symporters, possible effects of ethanol on the membrane potential as well as on membrane proton gradient should also be taken into account. This is the reason why higher inhibition constants are expected for proton symporters [van Uden, 1985]. The inhibition constants obtained for *C. halophila* fit well the values obtained before for facilitated diffusion type transporters (Table 3.4), and thus reinforces the idea of glucose being transported by a facilitated diffusion type system.

Proton-symport mechanisms have been claimed to display a characteristic pH dependence of transport velocity which decreases as ΔpH decreases at higher extracellular pH values [Höfer and Nassar,

1987]. A distinctly different pattern of pH dependence is usually obtained for the influx of substrate through a facilitated diffusion type transport system [Höfer and Nassar, 1987]. In this case, substrate influx may increase slightly, remain steady or decrease steadily for pH up to 7 or 8 [Höfer and Nassar, 1987]. The variation of *C. halophila* glucose uptake V_{max} at several extracellular pH was studied (Figure 5.3). Glucose uptake increases from pH 3 to 4 and remains approximately constant up to pH 7.

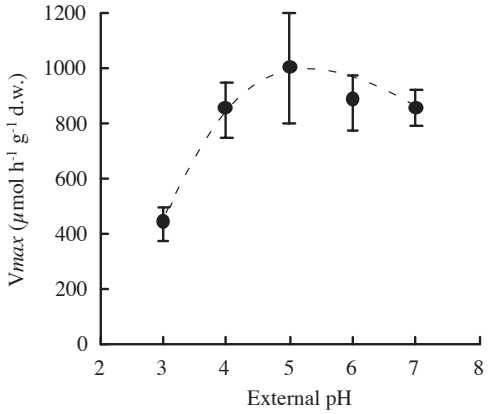


Figure 5.3. Glucose uptake maximal velocity as a function of pH of the cell suspension in cells of *C. halophila* grown in MM at 30ºC with 2% (w/v) of glucose as carbon source. Assays were performed with Tris-citrate buffer at different pH values.

Glucose facilitated diffusion in *D. hansenii* also revealed to be unaffected by external pH, glucose uptake velocity remaining constant at pH from 3 to 7 [Nobre *et al.*, 1999]. The same authors verified a slight decrease in V_{max} for pH below 5, for the glucose and xylose proton symporters. In the case of the symport H^+-maltose in *S. cerevisiae*, the V_{max} highly decreased for pH lower than 4 and higher than 6, remaining steady between 4 and 5.5 pH range [Loureiro-Dias and Peinado, 1984]. Transporters pH dependence is thus not straightforward among different yeasts and different compounds, since it is influenced not only by ΔpH but also by ΔΨ, and the contribution of these is different in each yeast and for each compound according to the mode of transport used.

The specificity of glucose transport was assayed using the most probable compounds that could eventually share the glucose facilitator such as other sugars and polyols. This was assayed measuring glucose uptake V_{max} in the presence of 100 mM of each compound (Table 3.5). According to Table 3.5, only mannose and 2-dexyglucose appear to be able to share the same transporter protein as glucose. For the case of the substrates which did not affect glucose uptake, higher concentrations were tested, up to 500 mM. Still no effect was observed. Since glucose is rapidly phosphorylated and metabolized by the cell, it is important to be able to the use non metabolizable compounds that share the same transporter to properly characterize transport and circunvey the interference of metabolism in uptake studies. Nevertheless, the use of this type of compounds can provide only quantitative information, since kinetic parameters may eventually be different from those for the natural substrates. In *C. halophila*, since 2-deoxyglucose, as a potential glucose non-metabolized analogue, did not inhibit glucose uptake further than 40%, it was not tested in order to verify whether inhibition was competitive and was not further used.

Table 3.5. Percentage of inhibition of initial uptake rates at the maximal velocity range of glucose uptake in *C. halophila* cells grown at 30ºC in MM with 2% (w/v) of glucose as carbon source. Cells suspension was incubated 2 minutes in the presence of 100 mM of each compound and then assayed for glucose transport in the range of maximal velocity.

Compound	% of inhibition of [^{14}C]-glucose uptake V_{max}
D(+) Fructose	No inhibition found
D(+) Mannose	52
2-Deoxyglucose	40
Glycerol	No inhibition found
D(-)Mannitol	No inhibition found

Glucose transport specificity varies between yeasts, as can be seen in Table 3.6. Actually, sugar and polyols are quite widespread in nature, and the share of the same transporter by several compounds might be advantageous for the colonization of certain environments. Moreover, the lack of specificity of each transporter might allow the competition, in mixed cultures, between different yeasts, like for example in the last stage of wine making where low amounts of different but important compounds are found [Postma *et al*., 1989]. Low-affinity uptake of facilitated diffusion type often accepts fructose in addition to glucose (Table 3.6). The type of glucose transport used by yeasts has been connected with their essential types of metabolism, *i.e.*, aerobic, anaerobic, fermentative or respiratory [van Urk, 1989b].

Table 3.6. Examples of glucose transport specificity in yeasts.

Yeast	Glucose transport type	Compounds which share the transport	/ References
C. shehatae	H^+-glucose symporter	Mannose	Lucas, 1987
	Facilitated diffusion (LA)	Xylose, mannose	
D. anomala	Facilitated diffusion		Gerós, 1999
	High affinity	Galactose	
	Low affinity	Fructose	
D. hansenii	H^+-glucose symporter	Mannose	Nobre *et al*., 1999
	Facilitated diffusion (LA)	Xylose	
K. lactis	Facilitated diffusion (LA)	Fructose	Chen *et al*., 1992
K. marxianus	H^+-glucose symporter	Galactose	Gasnier, 1987
	Facilitated diffusion (LA)	Fructose	
S. cerevisiae	Facilitated diffusion	Fructose, mannose	Diderich *et al*., 1999
S. pombe	H^+-glucose symporter	Fructose	Höfer and Nassar, 1987; Heiland *et al*, 2000

* LA-Low affinity

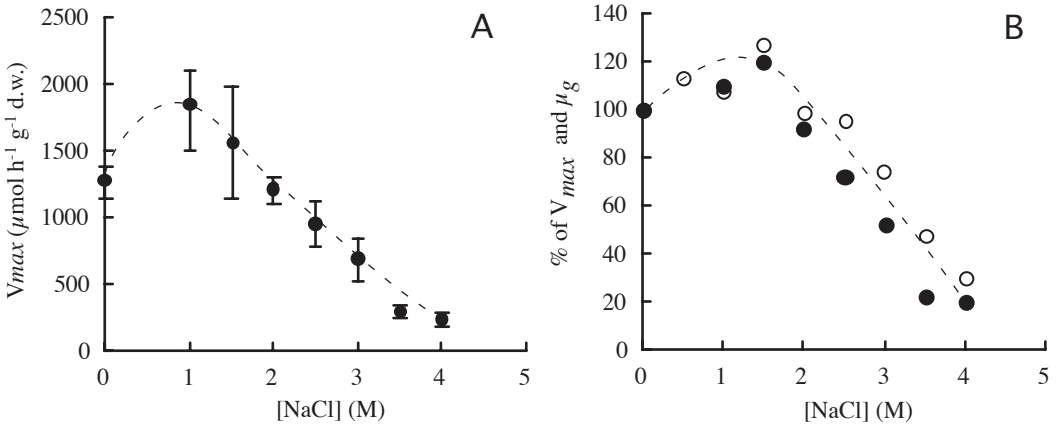
Van Urk (1989b) found a correlation between the presence of high-affinity glucose transport (H^+-symporter) systems and the absence of aerobic fermentation upon addition of excess glucose to

steady-state cultures. In contrast, the yeasts that did shown aerobic alcoholic fermentation during glucose pulse experiments, such as *S. cerevisiae*, *Torulopsis glabrata* and *S. pombe* presented exclusively low-affinity facilitated-diffusion carriers. As pointed above in Chapter 2, *C. halophila* is a respiro-fermentative yeast, and thus the glucose uptake of facilitated diffusion type characterized seems to fit well with this observation.

Effect of salt over glucose transport

Considering that the effects of salt on growth rate of *C. halophila* presented in Chapter 2 could have a counterpart in membrane transport of the carbon source, as a highly resistant yeast *C. halophila* was assayed for the variation of the maximal velocity of glucose transport in the presence of increasing salt concentrations in the medium (Figure 3.9). As can be seen, glucose uptake increases up to 1.5M NaCl and decreases progressively for salt concentrations above 2M NaCl. When the values of sugar uptake were compared with the results on specific grown rate in the same concentration (Figure 3.9-B), it became clear that the growth sensibility to salt stress accompanied closely the glucose uptake under the same culture conditions. Whether the limitation of growth is responsibility of glucose uptake alone remains to be further elucidated.

Figure 3.9. A- Initial uptake rates at maximal velocity of labelled glucose as a function of salt in the medium, **B-** Comparison between the specific growth rate (μg) (○) and glucose uptake maximal velocity (●) in terms of percentage. *C. halophila* cells were grown in MM at 30ºC with 2% (w/v) of glucose as a carbon source and in the presence of different salt concentrations and assayed with the same amount of salt in 100 mM Tris-citrate buffer, pH 5.0.



At high salt concentrations, it is likely that glucose uptake may limit growth, since, as pointed above (Section 3.1.1), salt reduces the permeability of plasma membrane and turns it more rigid, which may have implications in normal functioning of transport proteins and consequently in solutes uptake through these proteins. Curiously the growth rate enhancement up to 1.5M of NaCl was accompanied by sugar uptake V_{max} (Figure 3.9-B). *D. hansenii* is also a yeast in which growth rate

showed a slightly increase at low salt concentrations [Prista *et al*., 1997]. Sugar uptake of H^+-symport type published by Nobre and collaborators (1999) has been shown to be dependent on the presence of salt. Uptake upon glucose addition under the same experimental conditions as *D. hansenii* [Nobre *et al*., 1999] was assayed in *C. halophila* cells. As before, extracellular alkalinization upon glucose addition to a cell suspension incubated on salt was not observed. Instead, strong acidification, indicating indirectly the function of an H^+-ATPase was observed (not shown). It has been reported that hyperosmotic stress reduces glucose consumption and uptake rate in *S. cerevisiae* and in the halotolerant yeast *D. hansenii* [Singh and Norton, 1991; Neves *et al*., 1997; Loray *et al*., 1998]. Furthermore, Türkel (1999) also reported that hyperosmotic stress repress the transcription of *HXT2* and *HXT4* genes encoding for high and intermediate affinity glucose transporters in *S. cerevisiae*. Repression of these genes was affected, not only by 1M of NaCl but also by 1.5M of glucitol, which points more to an osmotic effect than a direct toxic effect caused by sodium ions. The modes of transcriptional repression of these genes by salt are not yet clear, but it is conceivable that hyperosmotic stress might modify the activities of the transcriptional regulators of *HXT2* and *HXT4* through the HOG signal transduction pathway or through an interaction of several signal transduction pathways. Thus, the effects of NaCl in solutes uptake cannot be resumed to a membrane effect, but implies regulation at molecular level. Furthermore, membrane alterations in stress conditions may be sensed by the cell and trigger repression or activation at molecular level of several genes which may, in turn, induce physiological alterations. The extent of glucose uptake modulation in salt stress conditions is poorly studied. It is obviously necessary to acquire much more knowledge in order to unravel the mechanism underlying the transport of solutes and their implications in yeast survival in salty environments.

3.2.2.2. *Glycerol uptake study*

Glycerol uptake in *C. halophila* was assayed in cells growing on different carbon sources (Figure 3.10). For the particular case of glucose, two different concentrations were used, 2% and 0.1%, corresponding to, respectively, repression and de-repressing concentrations (Figure 3.10). As can be seen, glycerol uptake measured points to glycerol being transported through a permease following Michaelis-Menten kinetics. *C. halophila* kinetic parameters (Table 3.7) are different according to the carbon source. Although not clearly repressed by glucose, glycerol transport is somehow regulated by the amount of this sugar, since cells grown in 0.1% (w/v) glucose present glycerol uptake V_{max} increased in approximately 40%, and the affinity duplicated (Figure 3.10; Table 3.7).

Active transport for glycerol of the H^+-symport type has been described in a large number of different yeasts [Lages *et al.,* 1999] and extensively characterized in *D. hansenii* [Lucas *et al*., 1990], *P. sorbitophila* [Lages and Lucas, 1995] and *S. cerevisiae* [Lages and Lucas, 1997] (Section 3.1.2.2). Thus, in order to elucidate the type of mediated transport *C. halophila* was using we searched for external alkalinization following a glycerol pulse. Proton uptake kinetics was determined in glucose growing cells in the presence and in the absence of 1M NaCl (Figure3.11, Table 3.8). The proton uptake kinetics obtained were compared with those of glycerol uptake under the same conditions (Figure 3.12; Table 3.8). Results presented in Figures 3.11 and 3.12 show two different situations. By one side the K_m of cells assayed with and without 1M NaCl, for both proton and labelled glycerol, uptake was similar (Table 3.8). On the other hand, V_{max} variations were quite important when we compare proton and labelled glycerol uptake (Table 3.8). While the presence of proton uptake upon glycerol addition *de per se* could indicate the functioning of glycerol transport as an H^+-symport, the absence of an accepTable stoicheiometry is

apparently not consistent with this hypothesis. The possibility of glycerol being permeated through two different systems functioning simultaneously, a symporter and a lower affinity transport, could explain the higher V_{max} of labelled glycerol in comparison to protons. Nevertheless, applying Graph Pad Prism non-linear regression analysis to these data, the presence of two mediated systems was not suggested.

Figure 3.10 Michaelis-Menten (**A)** and Eadie-Hofstee (**B)** plots of initial uptake rates at pH 5.0 of [^{14}C]-glycerol by cells of *C. halophila* grown in MM with different carbon sources - Glucose 2% (w/v) (●), 0.1% (w/v) (○); Glycerol 2% (w/v) (■); Mannitol 2% (w/v) (▲); Ethanol 2% (w/v) (□).

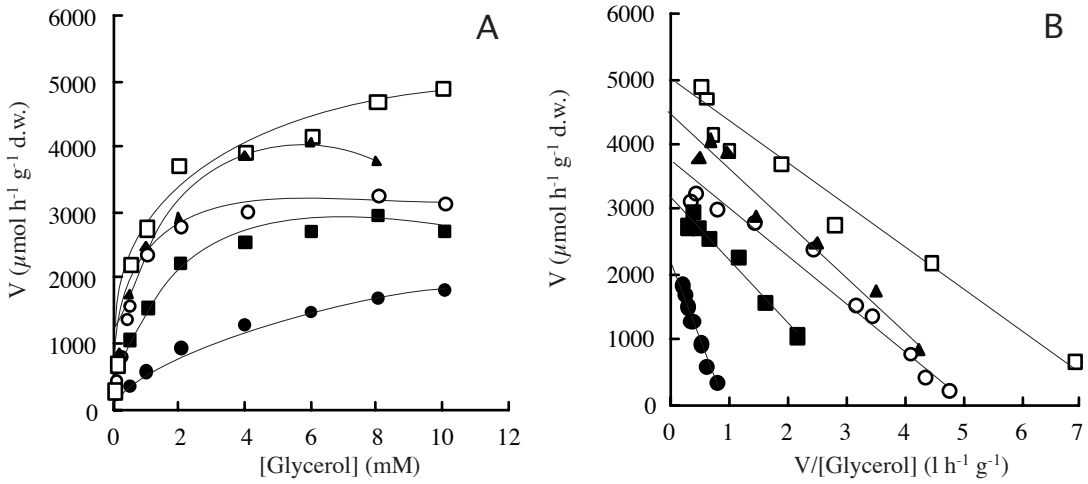


Table 3.7. Kinetic parameters of the initial uptakes rates of labelled glycerol in cells of *C. halophila* grown in MM supplemented with 2% (w/v) of different carbon sources. Data obtained by non-linear regression analysis using GraphPad Prism software.

Carbon source	**Km** (mM)	**Vmax** (μmol h^{-1} g^{-1} d.w.)
Glucose 2% (w/v)	1.41 ± 0.43	2590 ± 200
Glucose 1% (w/v)	0.68 ± 0.05	3600 ± 100
Glycerol	1.38 ± 0.30	3420 ± 130
Mannitol	0.68 ± 0.23	3820 ± 1000
Ethanol	0.52 ± 0.04	4200 ± 440

Figure 3.11. Michaelis-Menten (**A**) and Eadie-Hofstee (**B**) plots of initial uptake rates at pH 5.0 of protons upon glycerol addition in the absence (●) and in the presence (○) of 1M of NaCl, by cells of *C. halophila* grown in MM supplemented with 2% (w/v) glucose.

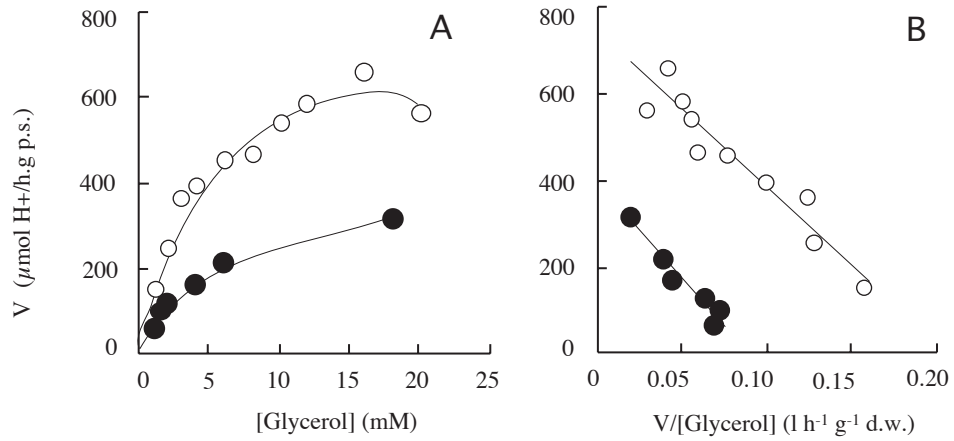
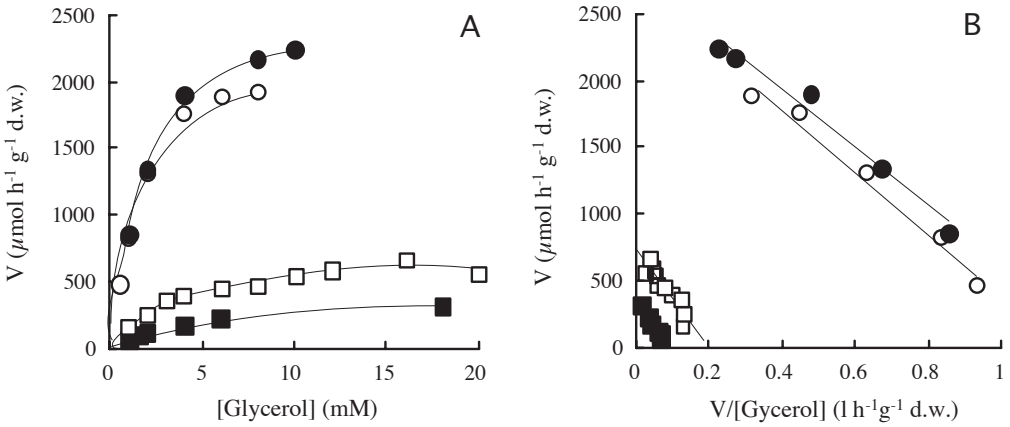


Figure 3.12. Michaelis-Menten (A) and Eadie-Hofstee (B) plots of initial uptake rates at pH 5.0 of [^{14}C] glycerol (●,○) and protons (■,□) upon glycerol addition in the absence of salt (closed symbols) and in the presence of 1M of NaCl (open symbols) by cells of *C. halophila* grown in MM supplemented with 2% (w/v) glucose.



The lack of 1proton/1substrate molecule stoicheiometry might not be indispensable to characterize a transport system as a symporter. For example, Lages and Lucas (1995) found a stoicheiometry value of 1 for the glycerol-H^+ symporter of *P. sorbitophila*, but this varied with salt in the growth medium and in the assay. On contrary, Nobre and Lucas (1999) did not find a

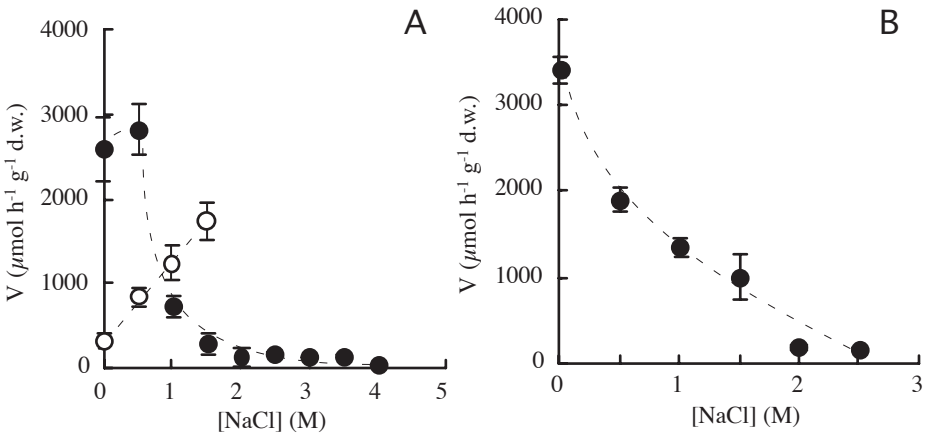
stoichiometric value of 1for the inducible glucose-H^+ symporter of *D. hansenii*, unless assays were performed in the presence of, at least, 1M NaCl.

Table 3.8. Kinetic parameters of the initial uptakes rates of labelled glycerol and proton uptake in cells of *C. halophila* grown in MM supplemented with 2% (w/v) of glucose and assayed in the absence and in the presence of 1M NaCl. Data obtained by non-linear regression analysis using GraphPad Prism software.

Conditions	***Km*** (mM)	***Vmax*** (μmol h^{-1} g^{-1} d.w.)
Uptake of [^{14}C] glycerol without salt	1.41 ± 0.43	2590 ± 200
Uptake of [^{14}C] glycerol with 1M of Nacl	1.21 ± 0.41	1926 ± 325
Proton uptake without salt	1.69 ± 0.05	319 ± 73
Proton uptake with 1M of NaCl	1.49 ± 0.49	827 ± 178

Labelled glycerol and proton uptake parameters were subsequently determined in *C. halophila* cells grown in the presence of increasing salt concentrations, as reported before for *D. hansenii* [Nobre *et al.,* 1999] and stoicheiometry was compared (Figure 3.13). Results show a variation of labelled glycerol V_{max} inverse to the variation of proton uptake V_{max}. A stoicheiometry of 1/1 is approximately reached in cells cultivated between 500 mM and 1M NaCl. The steep decrease observed for labelled glycerol V_{max} could eventually relate negatively with intracellular glycerol concentrations observed in cells cultured in the presence of increasing salt concentrations (Chapter 2).

Figure 3.13. Initial uptake rates of [^{14}C]-glycerol (●) and protons (○) at pH 5.0 in *C. halophila* cells grown in MM with different salt concentrations supplemented with **A-** 2% (w/v) glucose and **B-** 2% (w/v) glycerol. Proton uptake was not assayed in glycerol grown cells.



A decrease, though not so steep, was also observed for labelled glycerol uptake V_{max} on glycerol growing cells in the presence of increasing concentrations of salt (Figure 3.13-B). As in glucose growing cells these produce and accumulate high amounts of glycerol (not shown). This way, a possible explanation for these results would be a feed-back inhibition of glycerol uptake system by the substrate. This was postulated for the decrease in glycerol transport V_{max} in cells of *P. sorbitophila* grown in increasing salt concentrations [Lages and Lucas, 1995]. Nevertheless, the opposite increase in proton uptake V_{max} does not corroborate entirely this hypothesis. The inhibition of transport in bulk, as a result of an unfavourable chemical gradient of one of the co-substrates, should also correspond to the decrease in proton uptake. Yet, proton movements may also be affected by the ion gradient. Considering the need the cell experiences to export sodium ions at such high molarities of NaCl, the global ionic gradients must be affected by the work of sodium, potassium and proton pumps and/or antiporters. Finally, we cannot disregard the possibility of an experimental artefact, though initial uptake measurements at 10 sec are supposed not to be easily prone to these problems. On contrary to the glucose proton symport in *D. hansenii* [Nobre *et al.,* 1999], which proton uptake was observed only when the cells were shocked in salt, *C. halophila* alkalinization was observed in all circumstances. Thus, the two cases are most probably different in mechanism and biological function.

Glycerol passive diffusion was shown to exist in a large number of yeasts [Lages *et al.,* 1999]. As mentioned above, this was thought to be the *usual* way of glycerol permeating yeast plasma membrane. Nevertheless, recently it was shown that a diffusion of glycerol directly through the lipid bilayer would be rather difficult due to the chemical nature of this compound. Accordingly, it was shown by the same authors that in *S. cerevisiae* what was previously though to be passive diffusion through the lipids was actually dependent on the presence of the channel protein Fps1 [Oliveira *et al.,* 2003]. There is little evidence as to the existence of identical channels in other yeasts [Neves *et al.,* 2004]. Furthermore, it has been published that *S. pombe*, thought not possessing a gene identical to *FPS1* shows identical behaviour to *S. cerevisiae* Fps1p [Kayingo *et al.,* 2004]. It is still unclear whether this passive diffusion is due to the action of channels such as Fps1 or aquaglyceroporins, or instead is truly due to the diffusion across the lipid bilayer. Several attempts were made in order to unveil if passive diffusion was occurring simultaneously with glycerol-H^+-symport in *C. halophila* cells. Although with difficulty and with poor reproducibility we found that in *C. halophila* cells glycerol was entering simultaneously by a passive diffusion type system and by the glycerol-H^+-symport system (Figure 3.14).

Figure 3.14. Michaelis-Menten (**A**) and Eadie-Hofstee (**B**) plots of initial uptake rates at pH 5.0 of labelled glycerol in cells of *C. halophila* grown in MM with 2% (w/v) glucose as carbon source.

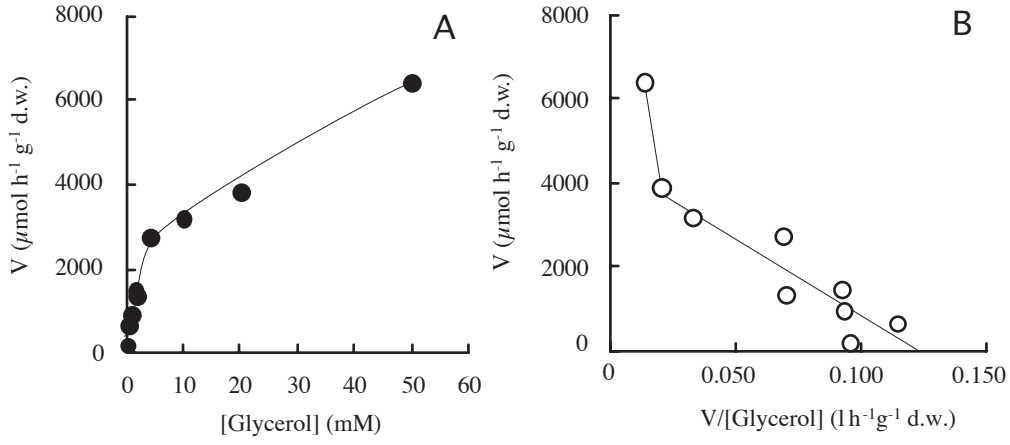


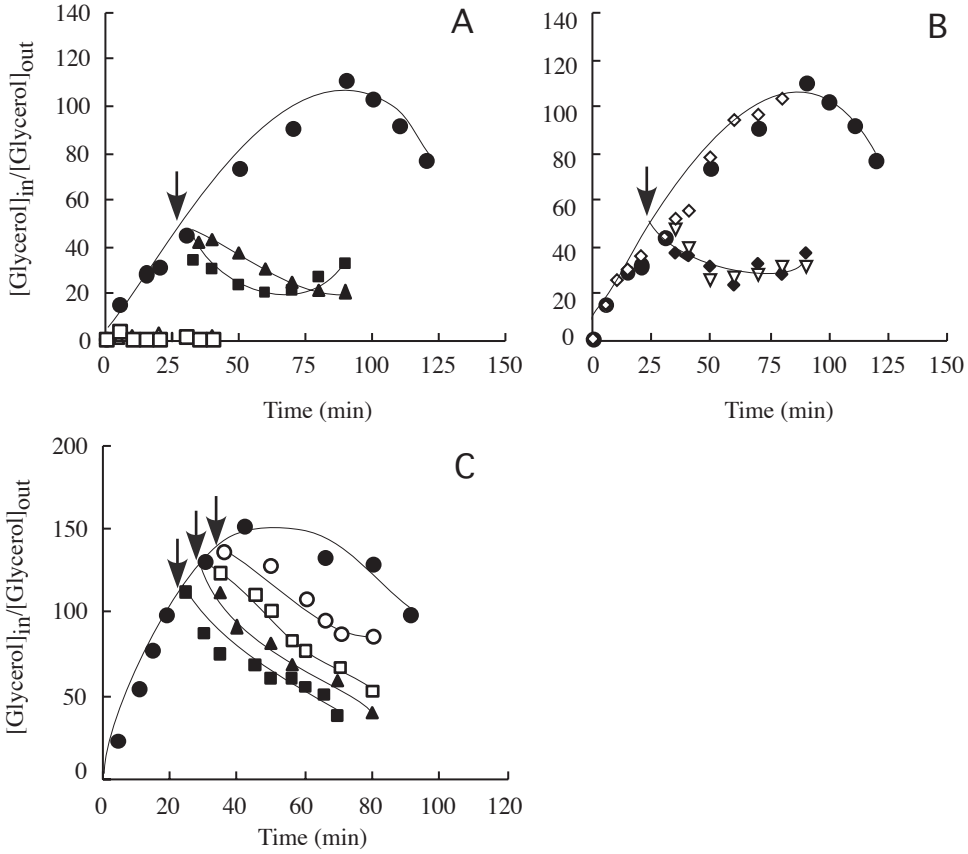
Table 3.9. Kinetic parameters of the initial uptake rates of labelled glycerol and proton uptake in cells of *C. halophila* grown in MM supplemented with 2% (w/v) glucose. Data obtained by non-linear regression analysis using GraphPad Prism software.

Kinetic Parameters	
Km (mM)	2.06 ± 0.71
Vmax (μmol h^{-1} g^{-1} d.w.)	3006 ± 456
Kd (l h^{-1} g^{-1} d.w.)	0.069 ± 0.011

This passive diffusion was only visible when we increased the glycerol concentrations and the incubation time used in the experimental assays. Figure 3.14 shows Eadie-Hofstee plots with biphasic kinetics according to the simultaneous passage of glycerol through a permease and a non-saturation, first order, kinetic branch. These results were confirmed by Graph Pad Prism non-linear regression analysis and the kinetic parameters thus obtained are presented in Table 3.9. Glycerol entry of passive diffusion type has also been observed in *P. sorbitophila* cells grown either in the absence and in the presence of different salt concentrations [Lages and Lucas, 1995]. Plasma membrane permeability is reduced in the presence of salt, and accordingly *P. sorbitophila* cells showed a decrease in passive diffusion constants with increasing salt concentrations in the medium [Lages, 2000].

Co-transport systems are essentially characterized by their ability to concentrate substrates against its chemical gradient between intra and extracellular media. This is achieved according to the energetic coupling of the co-transported ion gradient. For this reason, all the drugs which can act as uncouplers for ions or indirectly affect their concentration equilibrium, not only affect measurements, but should also affect the capacity of the transporter to create a thermodynamically unfavourable chemical gradient for the substrate. This can be seen either through the prevention of accumulation when cells are incubated in the presence of the drug or by inducing labelled substrate efflux when some of it has already accumulated, provided it has not been metabolised in the meantime. Accumulation of intracellular labelled glycerol was performed in *C. halophila* cells (Figure 3.15). Furthermore, glycerol efflux following the addition of several compounds, some of them uncouplers, was also performed (Figure 3.15). The uncoupling effects of CCCP and TPP were tested by incubating the cells with these drugs from the beginning of the accumulation assay, or by adding them after accumulation had started (Figure 3.15-A). CCCP and TPP had equal effects, preventing accumulation, while efflux corresponded to approximately 50% of the accumulated label (Figure 3.15-A). On the other hand, non-labelled substrates such as mannitol, galactose, xylose and sorbitol elicited a less significant label efflux and none of them led to a complete efflux of labelled glycerol (Figure 3.15-C). Additionally, glycerol-3-phosphate, supposedly the first compound produced by glycerol consumption, did not interfere with labelled glycerol accumulation (Figure 3.15-B). Cold glycerol as well as glucose elicited a partial efflux of the same dimension as the ionophores CCCP and FCCP (Figure 3.15 B). The efflux results as well as the lower maximum accumulation ratios could also derive from glucose and glycerol sharing the same transporter. Nevertheless, temptative assays to determine inhibition of glycerol uptake by cold glucose were inconclusive (not shown).

Figure 3.15. Accumulation ratios of [^{14}C]-glycerol in Tris-citrate buffer pH 5.0 at 30ºC, by cells *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose (●). **A-** Prevention (□,Δ) and efflux (■ ,▲) of labelled glycerol by the addition of 50 μM CCCP (squares) and 18 mM of TPP (triangles). **B-** Accumulation ratios of labelled glycerol in the presence of 100 mM of glycerol-3-phosphate (◊) and efflux of labelled glycerol by the addition of: 100 mM glucose (◆) and 100 mM glycerol (∇). **C-** Accumulation ratios of labelled glycerol in the presence of 100 mM of mannitol (○), galactose (■), xylose (□) and sorbitol (▲).



Glucose is a preferential carbon source compared to glycerol. In the presence of glucose, glycerol consumption generally does not happen [Lages and Lucas, 1997]. Measuring accumulation of radiolabelled glycerol in the presence of non-labelled glucose should give us a more realistic notion of how much intracellularly retained label actually corresponds to transport-driven accumulation of glycerol, by minimising the interference of glycerol metabolism in the accumulation measurements. Glucose prevented, to some extent, the accumulation of glycerol (Figure 3.16), being the maximum glycerol accumulation ratio attained in the presence of glucose approximately 20% of the value observed in its absence. This result is in accordance with the fact that glycerol is not simultaneously used with glucose as a carbon source in *C. halophila* cells (Chapter 2), and this may be suggested, as a consequence of glucose regulation over glycerol uptake. On the contrary, *P sorbitophila* did not present a reduction of glycerol accumulation in the presence of glucose, being glucose and glycerol

simultaneously dissimilated in this yeast [Lages and Lucas, 1995].

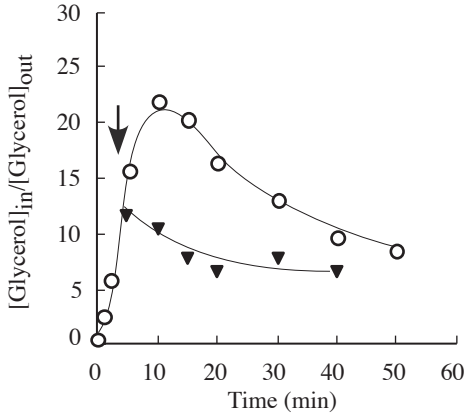


Figure 3.15. Accumulation ratios of [^{14}C]-glycerol in the presence of 100 mM of glucose (○) and efflux provoked by the addition of 100 mM of non-labelled glycerol (▼), in Tris-citrate buffer pH 5.0 at 30ºC, by *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose

Usually, if transport is reversible and the substrate is not immediately metabolised after entering the cell, complete efflux of labelled substrate is observed upon addition of an amount of the same non-radioactive substrate. In order to verify this hypothesis, since glycerol uptake reduced significantly in the presence of glucose, we determined and compared the intracellular concentration of labelled glycerol with those of non-labelled glycerol measured by HPLC (Figure 3.17).

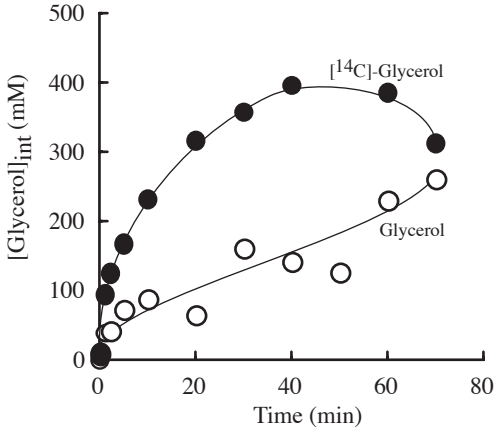


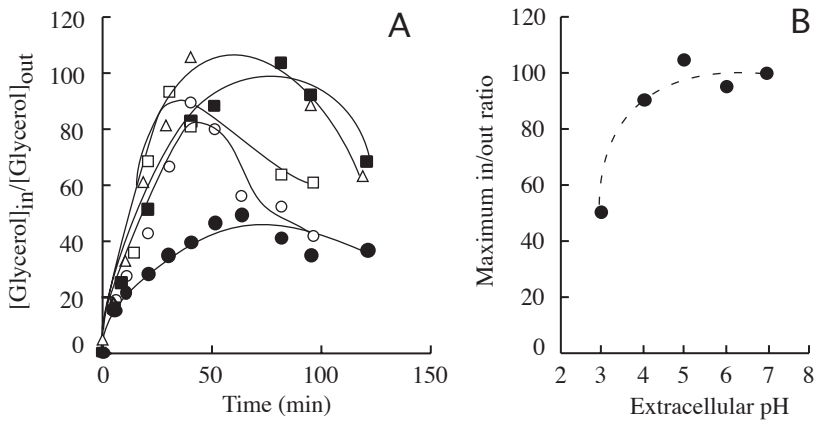
Figure 3.17. Intracellular concentration of glycerol, labelled (●), and non-labelled (○) of *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose at 30ºC.

As can be observed in Figure 3.17, in spite of the intracellular concentration of glycerol evidencing different behaviours, the final concentration (at 70 min) was similar in both processes. Thus, though this result points to the absence of metabolism interference in transport determination it is still unclear with the type of results obtained if metabolism is not in fact involved during the period of experiment (0-70 min). Irreversibility of the transport is the other possibility that may explain the

small efflux of labelled glycerol obtained with non-radioactive glycerol, CCCP and TPP. In fact, yeast symporters have been claimed to be unidirectional *in vivo* [Royt, 1983; Serrano, 1991; Stambuck *et al*., 1998]. In spite of that, van den Broek (1997) argued that the maltose permease in *C. utilis* is a reversible carrier acting in an asymmetrical way. Thus, the kinetics of inwardly moving maltose is different from that of outwardly moving maltose, leading to slow efflux compared with influx, namely in the presence of protonophores. Its likely that *C. halophila* H^+-glycerol symporter may function in a similar way as the maltose permease in *C. utilis*. Furthermore, as a halotolerant yeast it is probably more advantageous to the cell to accumulate glycerol and avoid leakage of glycerol to the medium. Nevertheless, we did not perform any additional studies in order to unveil the transports reversibility phenomenon.

Glycerol transport energetics was first attempted by the determination of the maximal values of labelled glycerol accumulation ratios over a pH range from 3 to 7 (Figure 3.18). Results in Figure 3.18. show a decrease in accumulation ratios for higher extracellular pH. These type of results were also obtained for the H^+-symporter of *P. sorbitophila* characterized by Lages and Lucas (1994, 1995), allowing to hypothesize that we are in the presence of an electrogenic symporter. If that is the case, accumulation ratios should accompany the variation of the ΔpH (difference between intra and extracellular pH) in the same range of pH values assayed.

Figure 3.18. A- Accumulation ratios of [^{14}C]-glycerol in *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose at 30ºC at different extracellular pH in Tris-citrate buffer, 100 mM. **B-** Maximal accumulation ratios at different pHs. Symbols: (●) pH 3, (○) pH 4, (■) pH 5, (□) pH 6, (△) pH 7.



We determined intracellular pH in cells incubated in buffer at different extracellular pH (Table 3.10) allowing us to determine ΔpH (Figure 3.19). Furthermore, we determined the influence of salt concentrations in internal pH. As can be observed in Figure 3.19 (Table 3.10), the internal pH is similar at all external pH assayed. The same was verified in the presence of several salt concentrations (Figure 3.19-B; Table 3.10), suggesting a very efficient and stable proton (ion) homeostasis, apparently independent of sodium gradients. In order to compare accumulation ratios with ΔpH, both values were

converted into mV units according to the reasoning in Section 3.1.3. (Figure 3.20). Accumulation ratios stayed approximately constant over the external pH range tested and did not accompany ΔpH variation. Since accumulation may be prevented by the action of either CCCP and TPP it is strongly suggested that glycerol transport depends simultaneously on both components of p.m.f.: ΔpH and ΔΨ.

Figure 3.19. Intracellular pH of *C. halophila* cells grown in MM with 2% (w/v) glucose in the absence (**A**) and in the presence (**B**) of different salt concentrations and assayed at different extracellular pH with the same salt concentration as the growth media. **Symbols**: (■) pH 3, (□) pH 4, (●) pH 5, (○) pH 6.

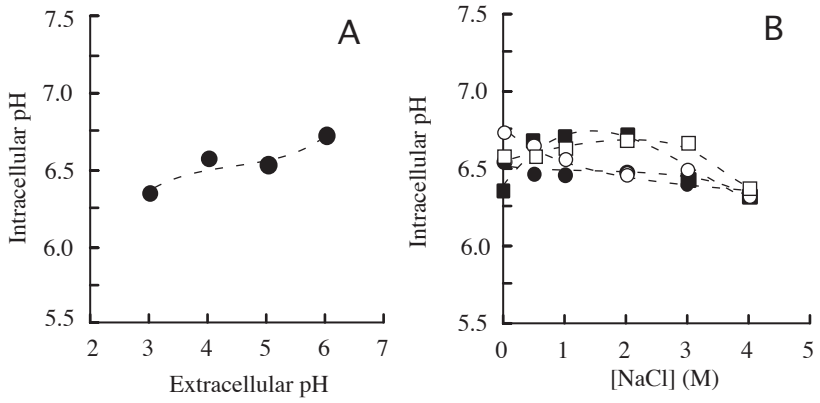


Table 3.10. Values of intracellular pH determined in *C. halophila* cells grown in MM with 2% (w/v) glucose in the absence and in the presence of different salt concentrations and assayed at different extracellular pH with the same salt concentration as the growth media.

	Extracellular pH			
[NaCl] (M)	**3**	**4**	**5**	**6**
0	6.36	6.58	6.54	6.73
0.5	6.68	6.58	6.45	6.66
1	6.72	6.64	6.45	6.57
2	6.71	6.68	6.46	6.48
3	6.43	6.66	6.39	6.49
4	6.32	6.37	6.32	n.d.

n.d. not determined

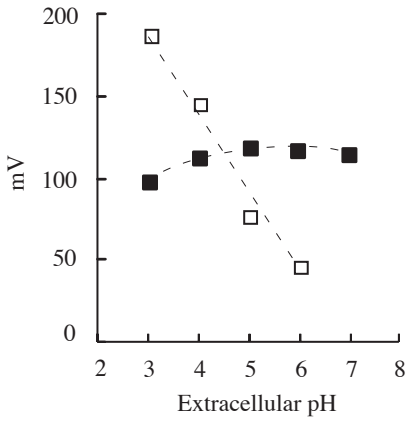


Figure 3.20. Effect of extracellular pH on labelled glycerol maximum accumulation ratios (■) and ΔpH (□) in 100 mM Tris-citrate buffer. ΔpH and accumulation values are related to mV units by a factor of 59.1 according to van den Broek *et al*., (1982) and de la Peña *et al*., (1982).

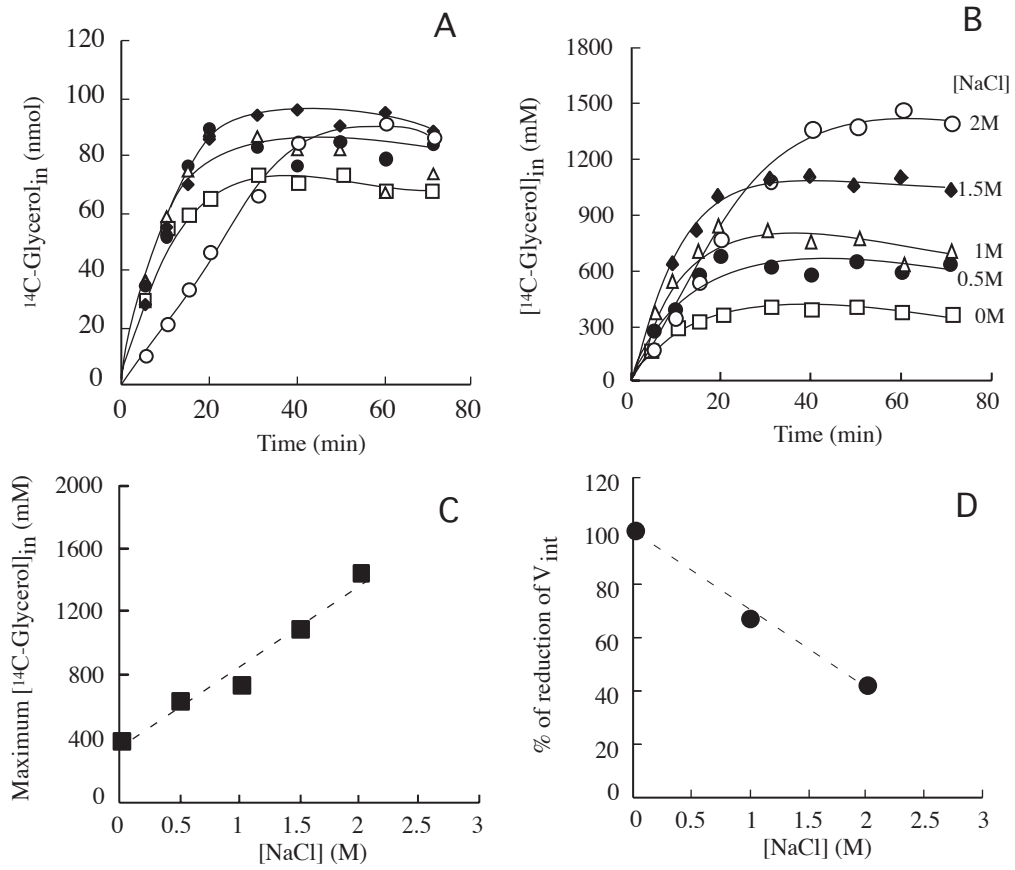
According to results in Figure 3.12, glycerol uptake was not stimulated by the presence of 1M of NaCl in the buffer. Similarly, we searched for the behaviour of glycerol accumulation in the presence of increasing salt concentrations in the buffer in *C. halophila* cells grown without salt (Figure 3.21). *C. halophila* accumulated approximately the same amount of glycerol in terms of internal moles in the absence as in the presence of salt up to 2M (Figure 3.21–A). When these values were indexed to the intracellular volume and since this decreased linearly for the same salt concentrations (Figure 3.21–D), an increase in intracellular glycerol molarity became evident (Figure 3.21–B). The maximum accumulation ratios obtained at increasing salt concentrations increased linearly with salt with an identical slope though of opposite sense as intracellular volume variation (Figure 3.21–C and D). Thus we may conclude that glycerol accumulation is not affected by salt stress and the increase observed in glycerol concentration with salt derived from intracellular volume changes. Considering that salt concentrations used (up to 2M) are not restrictive enough of *C. halophila* growth (Chapter 2), we cannot discard the possibility that higher salt stress conditions might lead to different results. In this stress range the accumulation of glycerol is compatible with the internal molarities measured before (Chapter 2), as well as with the unchanged specific growth rates (Chapter 2).

Characterization of glycerol transport proceeded with the determination of the specificity of the symporter. Several compounds, at the concentration of 100 mM, were tested as potential inhibitors of glycerol transport *Vmax* (Table 3.11). None of them has evidenced a significant inhibition (more than 30%). This led us to conclude that glycerol transport in *C. halophila* was highly specific for glycerol. Glycerol transport specificity is not surprising since, *C. halophila* was reported to be able to use as carbon sources a limited group of compounds (Chapter 2) and the glycerol symporters charcaterized so far were also shown to be specific for this compound [Lages and Lucas, 1995, 1997]. Moreover, the compounds in Table 3.11 also did not elicit proton uptake when assayed in glucose grown cells (not shown).

Several drugs, named protonophores are widely used to abolish more or less selectively the components of the p.m.f., as mentioned above. Proton gradients are widely accepted to be generated by the plasma membrane H^+-ATPase and thus we used some specific and unspecific inhitors of this pump in order to verify their indirect involvement in glycerol transport (Table 3.12.). As expected for a H^+-symporter, the protonophore CCCP highly inhibited glycerol transport, and nigericin, which permeabilizes preferentially the mitochondrial membrane [Kovác *et al*., 1982] of the cell for protons in

exchange of K^+ ions provoke a complete inhibition of glycerol uptake at the concentration used (Table 3.12.).

Figure 3.21. Intracellular accumulation of [^{14}C]-glycerol with time on Tris-citrate buffer, pH5.0 containing different salt concentrations, at 30ºC, in *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose. **A-** Moles of [^{14}C]-glycerol accumulated intracellularly. **B-** Concentration of accumulated [^{14}C]-glycerol. **C-** Variation of maximum intracellular glycerol concentration with salt in the buffer. **D-** Percentage of intracellular volume reduction at different salt conditions (cells shocked with salt). A and B have the same type of symbols.



As expected from the involvement of the H^+-ATPase in generating a proton gradient across membrane, DCCD and DESB inhibition percentage were high. As happened before for glucose transport *ortho*-vanadate provoked only a small inhibition over glycerol transport, perhaps due, as pointed before, by its unspecificity to the H^+-ATPase but also by the lower concentration used. In general, most of the inhibitors tested, had to be used in more concentrated solutions than in other yeasts such as *S. cerevisiae* [Lages, 2000], which may eventually be justified by a distinct composition of the plasma membranes of different yeasts.

TPP is an agent which was frequently used in the past to put in evidence the importance of

the electric potential in a specific transporter (Section 3.1.3). We found a considerable percentage of inhibition of glycerol transport provoked by the presence of this compound, which suggests that electric potential is implicated in the transport process. As said before in section 3.1.3, this compound has to be used carefully, namely at considerable concentrations like those we used. These were chosen because higher incubation times of lower TPP concentrations were not inhibitory. In order to access the involvement of the components of the proton motive force, CCCP and TPP inhibition over labelled glycerol *Vmax* at different pH were assayed (Figure 3.22).

Table 3.11. Percentage of inhibition of several compounds over glycerol uptake *Vmax* in *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose and assayed at pH 5.0 in Tris-citrate buffer 100 mM. 100 mM of each compound was incubated 2 minutes with cells prior to glycerol uptake measurement.

Compounds	**% of inhibition of [^{14}C]-glycerol uptake V_{max}**
D(+) Arabinitol	25
Erythritol	16
Ethyleneglycol	11
D(+) Galactose	27
D(+) Glucose	0
1,2-Propanediol	30
1,3-Propanediol	14
D(-) Mannitol	36
D(+) Mannose	31
D(-) Sorbitol	20
Xylitol	28
D(+) Xylose	19

100% V_{max} =2172 µmol h^{-1} g^{-1} d.w.

Table 3.12. Effect of different drugs on glycerol transport, expressed in percentage of reduction of *Vmax* of labelled glycerol uptake in *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose, and assayed at pH 5.0 in Tris-citrate buffer 100 mM. Each compound was incubated 2 minutes with cells prior to glycerol uptake

Drug	**Drug concentration**	**% of inhibition of [^{14}C] glycerol uptake Vmax**
CCCP	40 µM	**90**
Nigericin	0.1 µM	**100**
DCCD	2 mM	**99**
DESB	5 mM	**91**
Sodium *ortho*-vanadate	80 µM	**14**
TPP	18 mM	**76**

100% *Vmax* =2172 µmol h^{-1} g^{-1} d.w.

As can be seen in Figure 3.22., CCCP inhibited glycerol uptake at all pH assayed, while TPP increased their inhibition effect through the raising of the extracellular pH. These results suggest that while proton gradient is a prerequisite for glycerol transport, the electric potential only becomes important at higher pH values. Moreover, maximal glycerol uptake velocity showed a slightly decrease at higher pH, which is a typical behaviour of transporters depending on proton gradients such H^+-symporters. The sensitivity of glycerol transport to CCCP and TPP was already evidenced in the accumulation assays presented before. Thus, these results contribute to the characterization of glycerol transport as a H^+-glycerol symporter type system.

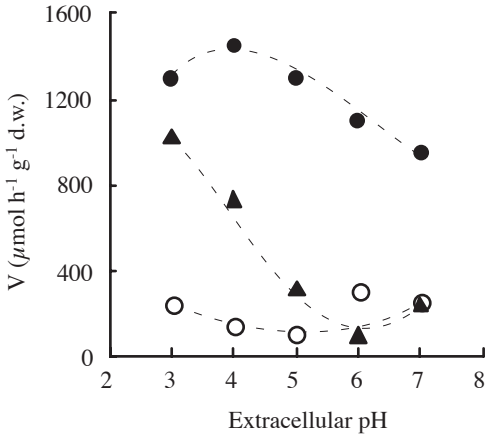
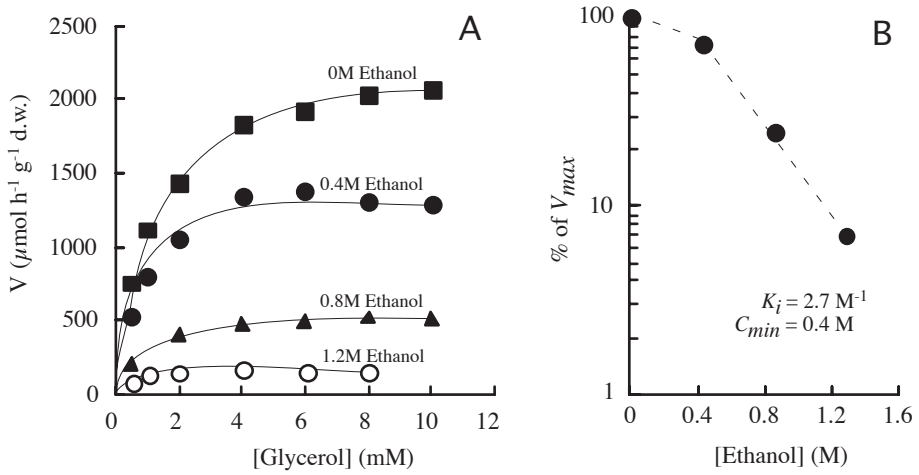


Figure 3.22. Effect of 50μM of CCCP (○) and 18 mM of TPP (▲) over the maximal velocity of labelled glycerol uptake (●) at different extracellular pHs in *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose at 30ºC.

Ethanol, inhibiting effect was also assayed (Figure 3.23), being experiments performed in the presence of increasing ethanol concentrations.

Figure 3.23. **A-** Effect of the presence of increasing ethanol concentrations over [^{14}C]-glycerol uptake in *C. halophila* cells grown in MM with 2% (w/v) glucose and assayed in Tris-citrate buffer 100 mM at pH 5.0., containing different ethanol concentrations. **B-** Percentage variation of glycerol uptake *Vmax versus* ethanol concentration.



Non-competitive inhibition was observed allowing the determination of a ethanol exponential inhibition constant (K_i) of 2.7 M^{-1} and a minimum inhibitory concentration (C_{min}) of 0.40 M. According to literature, K_i of proton symporters may vary substantially (Table 3.4). K_i values determined for the H^+-glycerol symporter of *C. halophila* situated between the range values corresponding to co-transporters. In accordance with the functioning of a symporter, which depends on the plasma membrane properties, and with the range (0.39-0.86) of values published for proton symporters [Lucas, 1987; Nobre *et al*., 1999], C_{min} value of *C. halophila* fitted those expected for proton-symporters.

The presence of labelled glycerol Michaelis-Menten kinetics; the alkalinization of media following a glycerol pulse and the determination of the respective proton uptake kinetics; the accumulation capacity against chemical gradient; the sensibility of glycerol uptake and accumulation to CCCP and TPP; the sensitivity to drugs affecting the H^+-ATPase; the high specificity of glycerol uptake; the sensitivity to ethanol according to parameters established for proton-symporters in glucose grown cells corroborates the characterization of glycerol uptake in *C. halophila* as constitutive, active and of H^+-symport type.

3.2.2.3. *Mannitol uptake study*

C. halophila growing on glucose produces and accumulates substantial amounts of mannitol, whose role in metabolism, though still unknown, is however clearly not directly associated with osmoregulation, as shown in Chapter 2. Furthermore, mannitol, like glucose and glycerol, is used by *C. halophila* cells at almost the same growth rate (Chapter 2). Uptake of labelled mannitol in *C. halophila* cells grown in different carbon sources was assayed (Figure 3.24) and followed saturation kinetics. The corresponding kinetics parameters are presented in Table 3.13.

Figure 3.24. Michaelis-Menten (**A**) and Eadie-Hofstee (**B**) plots of initial uptake rates at pH 5.0 of [^{14}C]-mannitol by cells of *C. halophila* grown in MM with different carbon sources: Glucose 2% (w/v) (○); Glycerol 2% (w/v) (■); Mannitol 2% (w/v) (△); Ethanol 2% (w/v) (●).

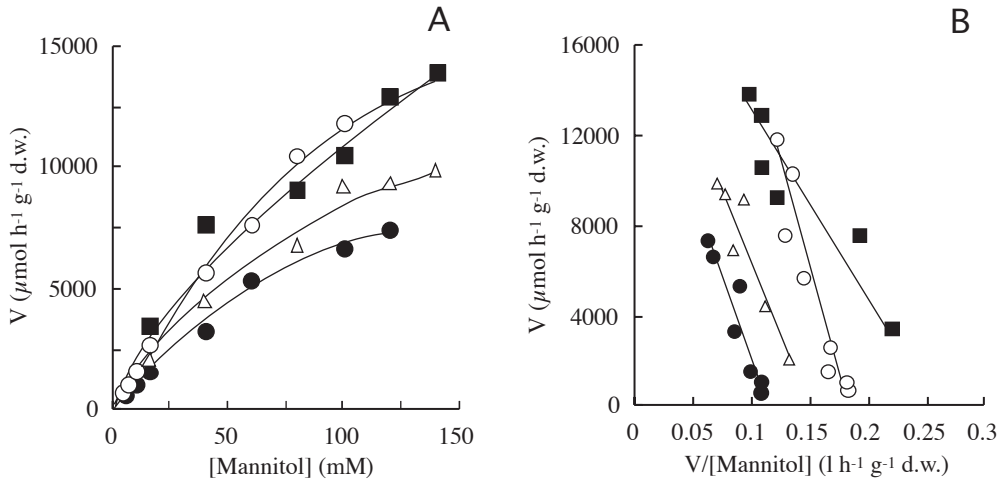


Table 3.13. Kinetic parameters of the initial uptakes rates of labelled mannitol in cells of *C. halophila* grown in MM supplemented with 2% (w/v) of different carbon sources. Data obtained by non-linear regression analysis using GraphPad Prism software.

Carbon sources	$\mathbf{K_m}$ (mM)	$\mathbf{V_{max}}$ (μmol h^{-1} g^{-1} d.w.)
Glucose	150 ± 61	21300 ± 9160
Glycerol	74 ± 5	20470 ± 990
Mannitol	126 ± 17	17330 ± 500
Ethanol	116 ± 29	15000 ± 700

As can be seen, though K_m values are higher than the ones determined for glycerol or glucose uptake (Tables 3.7 and 3.2), the extremely high V_{max} can guarantee an efficient supply of mannitol to the cell. This could explain why specific growth rates on either glucose, glycerol and mannitol are similar (Chapter 2). The results in Figure 3.24 and Table 3.13 show that mannitol transport, as glycerol and glucose transport systems, is constitutively expressed, although eventually not deprived of some degree of regulation. Since mannitol transport obeyed Michaelis-Menten kinetics we decided to search for the probable existence of extracellular alkalinization elicited by mannitol. Assays were performed in the absence and in the presence of 1M of NaCl in *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose with and without salt, but external pH changes were observed. Nevertheless results were not conclusive *de per se*, since mannitol is poorly soluble in water, and thus it was not possible to assay mannitol concentrations in the range of the K_m values determined for labelled mannitol uptake.

Active transport systems are characterized mainly by their ability to accumulate compounds against gradient. We tested the capacity of accumulation of the mannitol transport system (Figure 3.25). As can be seen mannitol is strongly accumulated againts its chemical gradient, suggesting that we are in the presence of an active transport system.

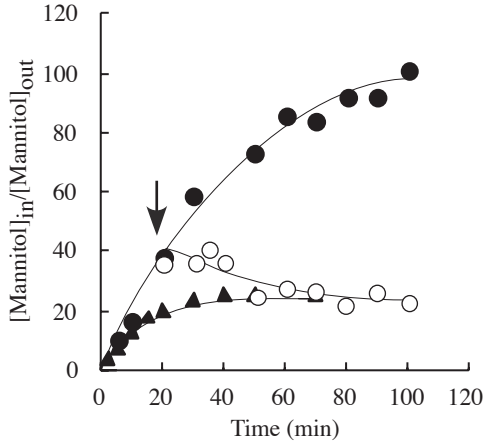


Figure 3.25. Accumulation ratios of [^{14}C]-mannitol in Tris-citrate buffer pH 5.0 at 30ºC, by cells *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose (●), efflux of labelled mannitol by the addition of 50 μM CCCP (○) and accumulation ratios in the presence of 100 mM of glucose (▲).

Similarly with what was found for glycerol transport, the efflux of labelled mannitol provoked by CCCP addition was partial and accumulation ratios in the presence of glucose were reduced (Figure 3.25).

We proceeded further with mannitol transport characterization and determined the responsiveness of this transporter to some drugs. Mannitol transport was found to be quite sensitive to the protonophore CCCP and to nigericin (Table 3.14), which points to the possibility of a proton gradient being involved in this uptake system. DCCD provoked a significant inhibition, which was not complemented by the other ATPase inhibitors (Table 3.14).

Table 3.14. Effect of different drugs on mannitol transport, expressed in percentage of reduction of *Vmax* of labelled mannitol uptake in *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose, and assayed at pH 5.0 in Tris-citrate buffer 100 mM. Each compound was incubated 2 minutes with cells prior to mannitol uptake measurement.

Drug	Drug concentration	% of inhibition of [^{14}C]-mannitol uptake V_{max}
CCCP	40 µM	70
Nigericin	0.1 µM	83
DCCD	2 mM	87
DESB	5 mM	40
Sodium *ortho*-vanadate	80 µM	34
TPP	18 mM	70

100% V_{max} = 21304 µmol h^{-1} g^{-1} d.w.

Since results concerning the use of inhibitors may be misleading, we consider these results as mere indications, which we further tried to support with different approaches. The TPP cation showed a percentage of inhibition similar to that found, using the same drug concentration, for the H^+-glycerol symporter (Table 3.12).

In order to determine the specificity of the mannitol transport system, some compounds were tested for their ability to inhibit labelled mannitol uptake (Table 3.15). None presented a percentage of inhibition that could be considered significant (Table 3.15). Thus, mannitol transporter, once more, seems to be specific for mannitol in the same way as glycerol transport for glycerol.

The effect of ethanol over mannitol uptake was also assayed. As expected, ethanol inhibited mannitol uptake following an exponential increase to increasing ethanol concentrations (Figure 3.26). The exponential inhibition constant (K_i) value of 1.4M is intermediate between the usual values for active transport and for simple diffusion [van Uden, 1985; Lucas, 1987, Nobre *et al*, 1999], but the minimum inhibitory concentration (C_{min}) of ethanol determined for mannitol of 0.43M, is almost the same found for glycerol transport (Figure 3.23).

Table 3.15. Percentage of inhibition of several compounds over mannitol uptake *Vmax* in *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose and assayed at pH 5.0 in Tris-citrate buffer 100 mM. 100 mM of each compound was incubated 2 minutes with cells prior to glycerol uptake measurement.

Compounds	% of inhibition of [^{14}C]-mannitol uptake *Vmax*
D(+) Arabinitol	28
Erythritol	13
D(+) Glucose	0
Glycerol	12
D(-) Sorbitol	27
Xylitol	22

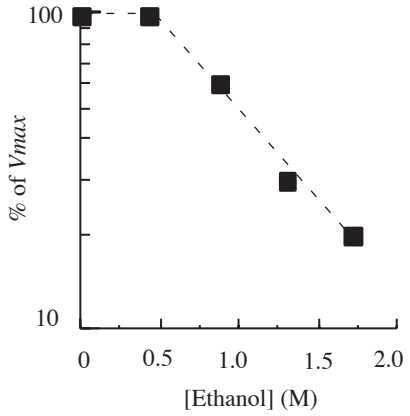


Figure 3.26. Percentage variation of mannitol uptake Vmax in the presence of increasing ethanol concentrations in *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose at 30ºC, and assayed in Tris-citrate buffer 100 mM at pH 5.0 containing different ethanol concentrations.

As mentioned above, another indirect evidence that might help to characterize a transport is the study of the dependence of the substrate uptake in relation to the medium pH. Identically to the H^+-glycerol symporter, mannitol uptake exhibits a pH-symporter type variation with extracellular pH (Figure 3.26), evidencing a reduction on V_{max} values for higher extracellular pH. It is clear, considering the same results obtained for glucose and glycerol transport, that the relation between extracellular pH and the type of transport used is not straitforward, since it is expected that proton-symporters will be visibly affected for higher extracellular pH values such as 7. Nevertheless, symporters are also dependent on $\Delta\Psi$, and this force varies most probably differently according to each yeast, thus influencing in a specific way the p.m.f.

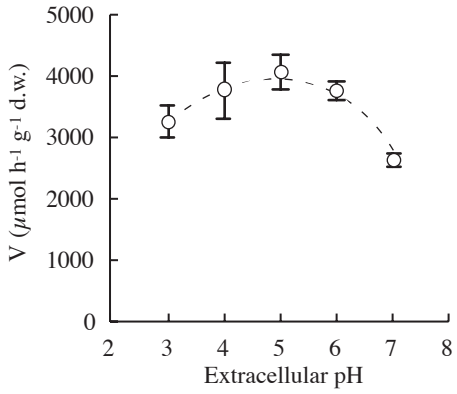
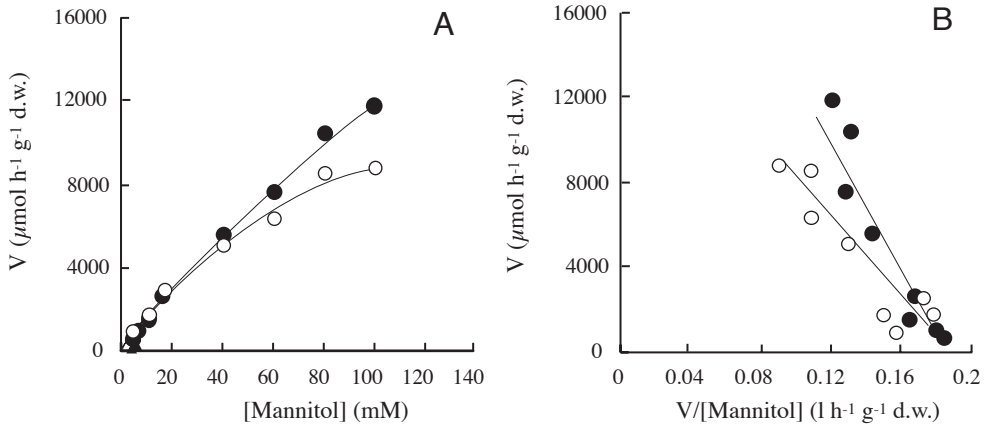


Figure 3.27. Labelled mannitol uptake at different extracellular pH of *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose at 30ºC. Assays were performed in Tris-citrate buffer 100 mM at pH ranging from 3 to 7.

Although evidences did not associate mannitol production with osmoregulation in *C. halophila*, we assayed mannitol transport in *C. halophila* cells shocked with 1M of NaCl (Figure 3.28) and in cells grown in the presence of increasing salt concentrations (Figure 3.29).

Figure 3.28. Michaelis-Menten (**A**) and Eadie-Hofstee (**B**) plots of initial uptake rates at pH 5.0 of [^{14}C]-mannitol in the absence of salt (●), in the presence of 1M of NaCl (○) by cells of *C. halophila* grown in MM supplemented with 2% (w/v) glucose.



As can be seen mannitol transport does not vary significantly in the absence and in the presence of 1M NaCl. On the other hand, results presented in Figure 3.29 concerning the mannitol transport of cells grown in the presence of increasing salt concentrations are compatible with the absence of an osmoregulation function with respect to mannitol synthesis in this yeast, since uptake decreases with increasing salt concentration. These results fit well with those obtained for intracellular mannitol determined in salt grown cells (Chapter 2), in which mannitol concentration is reduced when salt concentration in the growth medium increases. Since mannitol does not accumulate in response to

the presence of salt, the uptake measured is not affected by its intracellular concentrations as did glycerol uptake in cells growing in the presence of salt.

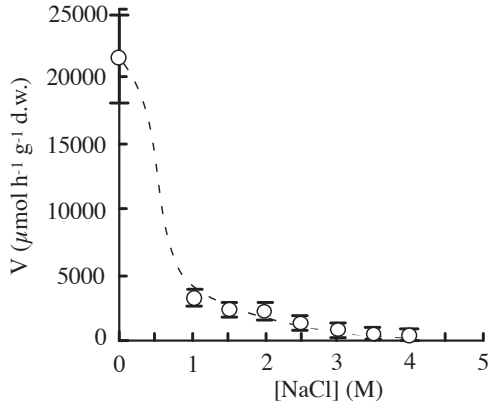
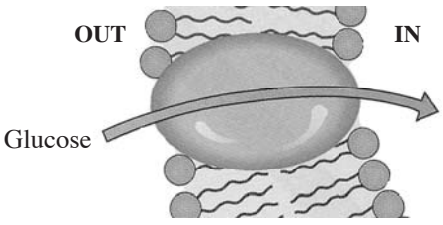


Figure 3.29. Initial uptake rates of [^{14}C]-mannitol at pH 5.0 in cells of *C. halophila* grown in MM supplemented with 2% (w/v) glucose with different salt concentrations.

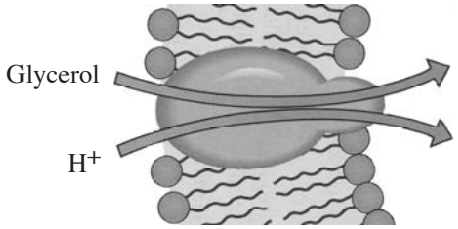
In spite of being impossible to measure any proton movements coupled to mannitol uptake and the affinity of mannitol being quite low, the accumulation curves presented, the sensitivity of uptake and accumulation to the presence of CCCP, the ethanol inhibition constants determined and the relation of mannitol uptake with external pH suggest altogether that this compound is actively transported through the plasma membrane of *C. halophila*.

4.3. Concluding remarks

According to the results presented in the sections above that aimed the characterization, as complete as possible, of glucose, glycerol and mannitol transport we may postulate that these compounds are using in *C. halophila* cells the following type systems:

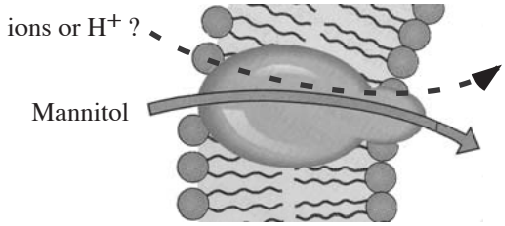


Facilitated diffusion
no proton uptake
low inhibition by CCCP
lower values of ethanol constant inhibition (K_i)
typical behavior with extracellular pH



H^+-Glycerol symporter

high affinity for glycerol
proton uptake associated with glycerol uptake
high inhibition by CCCP
concentrative capacity
higher values of ethanol constant inhibition (K_i)
dependence on extracellular pH
highly specific



Putative active transport of symport type

low affinity for mannitol
no proton uptake
high inhibition by CCCP
concentrative capacity
higher values of ethanol constant inhibition (K_i)
dependence on extracellular pH
highly specific