Chapter 2

C. halophila *kinetic growth assessment*

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Summary

Understanding yeast nutritional requirements and feeding strategies, together with the regulation of nutrient transport is important not only for successful cultivation in the laboratory but also for research and optimisation of industrial processes. This chapter presents physiological results concerning the growth parameters at constant temperature and the main metabolites produced in batch cultures of *C. halophila* growing in the absence and in the presence of salt. *C. halophila* is a slow growing yeast, although not severely affected by high salt concentrations. *C. halophila* cultures were grown in minimal medium at different initial medium pHs and using different carbon sources. The optimum medium pH range was 4 to 5, even in rich salt medium and for all the carbon sources tested. Intracellular solutes accumulated during growth were measured in cells cultivated on glucose, glycerol, mannitol and ethanol. Trehalose was found in all cases. Glucose and glycerol grown cells yielded mannitol and glycerol, while mannitol and ethanol grown cells yielded only mannitol. Specific growth rate in cells cultivated in the presence of increasing salt concentrations was not affected until 2.5 M of NaCl. At higher salt molarity, a progressive decrease in growth rate was observed. *C. halophila* was able to grow up to of 4.5M NaCl. Growth in the presence of 5M NaCl was still observed provided the inoculum was pre-adapted to salt presence. Glycerol proved to be the osmolyte, while the role of mannitol remains unknown. Oxygen consumption and CO_2 production were also measured in *C. halophila* cells growing on glucose mineral medium, and revealed that this yeast behaves, in batch cultures, as a respiro-fermentative organism. Both oxygen consumption and CO_2 production increased, although at different rates, in media with increasing salt concentrations.

2.1. Introduction

Yeasts are chemoorganotrophic, meaning they use organic forms of carbon for growth [Barnett, 1981, Barnett *et al*., 1990, 2000; Rose and Harrison, 1989]. Many and various compounds can be used as a source of carbon and energy by yeasts, glucose representing the easiest and the most widely used source of carbon followed closely by fructose, mannose and then galactose [Barnett, 1981; Rose and Harrison, 1989; Walker, 1998]. Glucose is the main sugar used in laboratory yeast culture, however may not be the most effectively metabolized sugar for all yeast, *e.g*. *K. lactis* grows faster in lactose than in glucose [Wésolowski-Louvel *et al*., 1996], neither is it freely available in natural environments. Its utilization in laboratory lays essentially in the fact that it has a general repressive and inhibitory effect on the assimilation of other sugars and some other compounds utilised by yeasts as carbon sources. The ability to use many different compounds as carbon and energy sources allows yeasts to colonize and proliferate in the most diverse niches from water, flowers and food to humans [Spencer and Spencer, 1997; Walker, 1998].

2.1.1. *Nutritional requirements particularly important in yeasts*

In general, yeasts have simple nutritional needs and are quite easy to cultivate. Most species can grow well in the presence of simple carbon and nitrogen backbone compounds together with inorganic ions and a few growth factors [Barnett, 1981; Rose and Harrison, 1989; Walker, 1998].

Nitrogen

Yeast cells have a nitrogen content of around 10% of their dry weight, and are capable of utilizing a range of different inorganic and organic sources of nitrogen for incorporation into the structural and functional nitrogenous components of the cell [Walker, 1998]. Although yeasts cannot fix molecular nitrogen, simple inorganic nitrogen sources such as ammonium salts are widely utilized as nitrogen source in yeast growth media, since it also provides a source of assimilable sulphur [Spencer and Spencer, 1996]. Some yeast can also grow on nitrate as a source of nitrogen, being nitrate assimilation ability used, as well as urea, for long time as a physiological discriminator between certain yeast genera [Hipkin, 1989]. A variety of other organic nitrogen compounds such as amino acids, peptides, purines, pyrimidines and amines can also provide the nitrogenous requirements of the yeast cells [Walker, 1998].

Mineral elements

Yeast requirements for minerals are similar to that of other cells with a supply of potassium, magnesium and several trace elements being necessary for growth. K^+ and Mg^{2+} are regarded as bulk or macroelements, which are required in millimolar concentrations to establish the main metallic cationic environment in the yeast cell [Walker, 1994; Rodríguez-Navarro *et al*., 1994; Walker, 1998]. Concerning potassium, the most prevalent cation in the yeast cytoplasm, yeasts have an absolute growth requirement for this mineral which is essential as a cofactor for a wide variety of enzymes involved in oxidative phosphorylation, protein biosynthesis and carbohydrate metabolism [Rodríguez-Navarro *et al*., 1994; Rodríguez-Navarro, 2000]. It is also involved in the uptake of other nutrients like phosphate, as a non-specific charge-balancer and as stabilizer of macromolecules and ribosomes. *D. hansenii* represents an

exception with relation to this element, since it may survive in media without potassium if sodium is provided instead [Prista *et al*., 1997], being this interchangeability between K^+ and Na^+ a unique feature of this yeast considering all the yeasts studied so far. Magnesium is also an absolute requirement for yeast growth and is present in cells at around 0.3 % of dry weight (representing concentrations in the mM range within cells), where it plays essential structural and metabolic functions [Walker, 1994].

Growth factors

Yeasts vary widely in their growth factor requirements [Koser, 1968; Spencer and Spencer, 1996]. These are organic compounds required in very low amounts for specific catalytic or structural roles in yeast, but are not used as energy sources [Koser, 1968]. Yeast growth factors include: vitamins (which serve vital metabolic functions as components of coenzymes), purines and pyrimidines, nucleosides and nucleotides, amino acids, fatty acids, sterols and other miscellaneous compounds [Koser, 1968; Walker, 1998]. When a yeast species is said to have a growth factor requirement this indicates that it cannot synthesise that particular factor, resulting in the impairment of growth and key metabolic processes until its addition to the culture medium be performed [Walker, 1998]. Most yeasts grow well in warm, moistly, sugary, acidic and aerobic environments. Those few species which prefer exceptional physical or chemical conditions are, nonetheless, very important in industry, often as spoilage organisms, and therefore their nutritional requirements must be carefully studied.

Oxygen

Yeasts are unable to grow well in the complete absence of oxygen. This is because, besides providing a substrate for respiratory enzymes during aerobic growth (terminal electron acceptor), oxygen is required for certain growth-maintaining hydroxylations such as those involving the biosynthesis of sterols and unsaturated fatty acids [van Dijken and Scheffers, 1986]. For instance, *S. cerevisiae* is auxotrophic for oleic acid and ergosterol under anaerobic conditions [Lagunas, 1986]. Oxygen should therefore be regarded as an important yeast growth factor and yeasts are frequently categorized into different groups with respect to their fermentative properties and growth responses to oxygen availability (Table 2.1).

Table 2.1. Classification of yeasts based on fermentative capacity [van Dijken and Scheffers, 1986; Sheffers, 1987; Gancedo and Serrano, 1989; Fiechter and Seghezzi, 1992; Walker 1998].

Class	Yeast examples	Effects
Obligatory fermentative	*Candida pintolopesii*	Naturally occurring respiratory-deficient yeasts. Only ferment, even in the presence of oxygen.
Facultatively fermentative		
Crabtree-positive	*Saccharomyces cerevisiae*	Alcoholic fermentation occurs in the presence of excess sugar under strictly aerobic conditions.
Crabtree-negative	*Candida utilis*	Under aerobic conditions no ethanol is formed and yeasts performing this way cannot grow anaerobically.
Non-fermentative	*Rhodotorula rubra*	No ethanol is produced either in the presence or absence of oxygen.

The influence of oxygen and sugar availability on yeast carbohydrate metabolism has been categorized under various regulatory phenomena, such as the Pasteur, Crabtree, Custers and Kluyver effects, which will not be approached under the scope of this thesis with exception of Crabtree effect (Table 2.1). Considering the destiny of the pyruvate arising from the conversion of glucose by the Embden-Meyerhof-Parnas pathway, yeasts may be divided in two groups: (i) obligate aerobes and (ii) facultative anaerobes [van Dijken and Scheffers, 1986; Sheffers, 1987; Gancedo and Serrano, 1989; Fiechter and Seghezzi, 1992]. Obligate aerobes are unable to utilize glucose in the absence of oxygen. Therefore their metabolism is exclusively respiratory, and pyruvate is channelled into the citric acid (TCA) cycle to be oxidized. This group, according to Gancedo and Serrano (1989), includes all species of the genera *Rhodotorula*, *Cryptococcus*, and some species of other genera such as *Torulopsis candida*, *Pichia fluxum, D. hansenii* and *Hansenula wingei*. Although *D. hansenii* has been reported as a respiratory yeast it may produce small amounts of ethanol under conditions of oxygen limitation [Neves *et al.*, 1997; Nobre, 2003]. Facultative anaerobes are able to utilize glucose under aerobic or anaerobic conditions. In the latter case they metabolise glucose to ethanol (the classical alcoholic fermentation). During aerobic growth both fermentation and respiration contribute to the catabolism of glucose. Depending on the magnitude of this contribution two more generic subgroups can be made: fermentative and respiratory yeasts [Gancedo and Serrano, 1989]. During aerobic growth of fermentative yeasts, respiration accounts for less than 10% of glucose catabolism. The rates of glucose catabolism tends to be very high (100 to 300 μmol glucose min^{-1} g^{-1} d.w.) and the rate of oxygen consumption low (5 to 50 μmol O_2 min^{-1} g^{-1} d.w.). This subgroup comprises yeasts of the genera *Saccharomyces* and *Brettanomyces* [Gancedo and Serrano, 1989]. The great majority of yeasts species belong to the subgroup of respiratory yeasts. During aerobic growth, less than 30% of the metabolised glucose is fermented. Respiration rate is very high (150 to 250 μmol O_2 min^{-1} g^{-1} d.w.) and catabolism slow (10 to 40 μmol glucose min^{-1} g^{-1} d.w.). Typical examples are species of the genera *Candida*, *Hansenula*, *Kluyveromyces*, some species of the genera *Torulopsis* and most *Pichia* [Gancedo and Serrano, 1989].

Distinction between fermentative and respiratory yeasts apply only to aerobic growth on glucose, fructose and mannose. The molecular basis to this rationale is related to the repression of several respiratory enzymes by the sugars in the group of fermentative yeasts [Gancedo, 1992, 1998]. With other carbon sources, repression of respiratory enzymes is diminished and respiration makes a great contribution to aerobic metabolism of such compounds by the fermentative yeasts [Gancedo, 1992, 1998].

pH

Yeasts generally grow well at initial culture medium pH between 4-6, but many yeasts are capable to grow over a wide pH range of 2 to 8 [Walker, 1998; Pitt and Hocking, 1997]. Usually they do not grow well at alkaline pH values, with exception of certain marine yeasts adapted to grow on alkaline seawater, as for example *D. hansenii* [Norkrans, 1966]. Most of the yeasts acidify their growth medium when growing actively due essentially to the action of the plasma membrane proton ATPase [Norkrans, 1966; Salhany *et al.*, 1975; Serrano, 1978, 1983; Borst-Pauwels, 1981; Eraso and Gancedo, 1987; Kotyk, 1994]. Although, extracellular pH variations, do not, usually have an impact in intracellular pH, since the cell has mechanisms that control efficiently internal proton homeostasis, indirectly regulating the uptake of a large number of nutrients and ions [Serrano, 1989].

2.1.2. *The roles of intracellular accumulation of polyols in yeasts*

The ability to produce and accumulate polyols (also termed sugar alcohol or polyhydroxy alcohols) is widely distributed in fungi, being glycerol, mannitol, arabinitol, erythritol and sorbitol the most frequent [Lewis and Smith, 1967; Brown and Simpson, 1990; Brown, 1972, 1976, 1978; Jennings, 1984; Brown *et al*., 1986; Pfyffer *et al*., 1986; da Costa and Nobre, 1989]. In fact, the formation of polyols is an integral part of the normal metabolism of various yeasts, but can be influenced by the conditions of growth [Rehm and Reed, 1981; da Costa and Nobre, 1989]. Though polyol production is common among yeasts, little is known about the function of polyols other than glycerol. In general suggested physiological roles for polyols include (i) osmoregulation as compatible solutes [Brown, 1972, 1978; Yancey *et al*., 1982; Jennings and Burke, 1990a; Luxo *et al*., 1993; da Costa and Nobre, 1989]; (ii) storage of reduced carbon and energy [Lee, 1969; Wang and Tourneau, 1972]; (iii) regulation of coenzymes [Loescher, 1987; Cioci and Lavecchia, 1994], and (iv) neutralization of hydroxyl radicals [Smirnoff and Cumbes, 1989; Wong *et al*., 1990; Chaturvedi *et al*., 1996; Perfect *et al*., 1996; Shen *et al*., 1997]. Evidence supporting these roles for polyols has been obtained primarily with fungi and animals, but are presently being studied actively in higher plants [Brown, 1978;Tarczynski *et al*., 1992].

Onishi (1960) has surveyed 119 strains of yeasts for polyol production and found that most species produced glycerol and arabinitol, and a few of them also small amounts of erythritol. A similar study for taxonomy purposes was made on 450 fungal species and in all fungi with exception of Oomycetes the polyols detected were glycerol, erythritol, ribitol, arabinitol, xylitol, sorbitol, mannitol and galactitol [Pfyffer *et al*., 1986, 1990; Pfyffer and Rast, 1989]. Fungi were divided into two large groups with respect to polyol production: (i) those that contained various polyols except mannitol (Zygomycetes and Hemiascomycetes) and, (ii) those that contained mannitol as well as other polyols (Chytidriomycetes, Euascomycetes, Basidiomycotina and Deuteromycotina).

Many physiological functions have been attributed to polyol accumulation, although the compatible solute function is by far the most studied among yeasts. Accumulation of compatible solutes (osmolytes), together with exclusion of the stress solute is a general mechanism by which microorganisms counteract the dehydration effects of diverse and fluctuating external solute compositions (Chapter 1, section 1.5). These compounds can be accumulated by endogenous production or by uptake from the medium to high concentrations, compensating the loss of turgor pressure, generating an intracellular surrounding that allow enzymes to work properly and stabilizing and protecting membrane structures [Brown, 1976, 1978; Brown and Simpson, 1972; Jennings, 1984].

Glycerol

As mentioned before in Chapter I, glycerol is the simplest alcohol with less effect in intracellular machinery at high concentrations. Therefore, it is the primary compatible solute most used by yeasts for cytoplasm a_w adjustment under salt stress. Besides, it is often accumulated by *S. cerevisiae* cell as a by-product when glucose or other easily fermentable sugars are converted to ethanol. The production of ethanol from glucose is a redox-neutral process, but the formation of biomass and oxidized by-products such as acetate generates an excess of reducing equivalents which can be disposed by the glycerol production through a NADH–consuming enzyme, thus restoring the redox balance, compensating for cellular reactions which produce NADH [van Dijken and Scheffers, 1986]. The production of glycerol seems to be absolutely essential for balancing cytoplasmic redox potential in the absence of oxygen [Ansell *et al*., 1997]. Glycerol is also a carbon and energy source under aerobic

conditions for most yeast, participating in the regulation of their metabolic activity. In *S. cerevisiae* glycerol is closely linked to the glycolytic flux regulation. A rapid increase in glycolytic flux may lead to the accumulation of phosphate intermediates such as hexoses phosphate, and consequently the inorganic phosphate pool reduced. Prosecution of glycolytic flux is then achieved by the recovery of the inorganic phosphate levels through the synthesis of trehalose or glycerol [Blázquez *et al*., 1994; Thevelein and Hohmann, 1995; Luyten *et al*., 1995; Nevoigt and Stahl, 1996]. Glycerol is also implicated in the synthesis of the lipid matrix of the plasma membrane in yeasts, where glycerophospholipids predominate. In fact, glycerol-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), compounds of the glycerol metabolic pathway, are the membrane phospholipids synthesis precursors [Daum *et al*., 1998]. Furthermore, glycerol dissimilation can contribute indirectly to the coupling of cytoplasmic to mitochondrial redox balancing through the glycerol-3-phosphate shuttle [Larsson *et al.*, 1998].

Mannitol

The role of mannitol has been studied in plants and fungi. In plants, the importance of polyols such as mannitol is reflected by the estimation that metabolism of these compounds, (rather than that of sugars), contributes to about 30% of the annual global primary carbon production [Tarczynski *et al*., 1992]. Mannitol is a major photosynthetic product and accumulates to high levels in several higher plant species, being detected in over 50 families. In spite of the functions of polyols in higher plants being still not very clear, a commonly held belief is that these compounds may confer beneficial traits on those species where they were found, rather than being simply intermediates of carbohydrate metabolism [Abebe *et al*., 2003]. Mannitol can function as an osmolyte accumulating during salt stress, like it does in the mushroom *Agaricus bisporus* [Stoop and Mooibroek, 1998]. Additionally it can also serve as a growth regulator supplying $NADP^+$ for the oxidation of the pentose phosphate shunt through mannitol NADPH dehydrogenase [Stoop and Mooibroek, 1998]. Mannitol is a common carbohydrate reserve material in many fungi, being accumulation of free mannitol characteristic of some *Aspergillus* strains [Lee, 1967; El-Kady *et al*., 1994], *Eurotium* [El-Kady *et al*., 1994], *Fennellia* [El-Kady *et al*., 1994], *Geotrichum* and *Endomyces* [Luxo *et al*., 1993]. In these two last fungi mannitol is accumulated in response to osmotic stress instead of glycerol, thus having an osmoregulatory function.

Mannitol has not been often directly associated with osmoregulation in yeasts, but rather with oxidative stress protection like it has been postulated for the pathogenic *C. neoformans* [Perfect *et al.*, 1996]. This yeast produces mannitol in culture and in infected animals being suggested that during the proliferation process in mammalian tissue, it acts as a scavenger of hydroxyl radicals, thus protecting against lethal oxidative stress mediated by host phagocytes [Wong *et al*., 1990; Chaturvedi *et al*., 1996; Perfect *et al.*, 1996]. According to Niehaus and Flynn (1994) *C. neoformans* produces mannitol at the expense of carbon sources such as glucose, fructose and mannose and thus the enzymes from the biosynthetic and the catabolic pathways were constitutively expressed. *C. albicans*, another yeast with recognized pathogenecity, is able to use mannitol as carbon source, meaning that it possess all the enzyme machinery necessary for that purpose, such as a NAD-linked mannitol dehydrogenase and also a specific transporter protein [Niimi *et al*., 1986]. Strains of *S. cerevisiae* differ in their ability to assimilate mannitol, but several industrial strains were reported as growing well with mannitol as carbon source [Quain and Boulton, 1987]. The same authors reported that aerobic respiration was absolutely essential for mannitol metabolism and in the presence of oxygen an NAD-dependent mannitol dehydrogenase necessary for mannitol utilization was detected. The function of mannitol as an acyclic hexitol

scavenger has been also demonstrated in a strain of *S. cerevisiae* unable to produce mannitol and genetically modified by the inclusion of a mannitol-1-phosphate dehydrogenase originating from *Escherichia coli* [Chaturvedi *et al*., 1997]. The inclusion of this enzymatic step allowed mannitol production, and guaranteed the new strain protection against oxidative and salt stress [Chaturvedi *et al*., 1997]. Furthermore, in recent articles it has been suggested that in *S. cerevisiae* mannitol can substitute for glycerol as an osmolyte [Shen *et al*., 1999], though this possibility has not yet been further addressed.

Extensive studies have been carried out on the production of polyols, such as glycerol, erythritol, xylitol, D-arabitol and D-mannitol, during the fermentation of soy-sauce by halotolerant yeasts, but no clear osmoregulatory function nor other type of functions has been attributed to mannitol [Onishi and Suzuki, 1966, 1968, 1969a,b, 1970a,b; Jennings, 1984]. However, more recently, it has been shown that *Z. rouxii*, besides glycerol and arabinitol, which has been shown to be used for osmoregulation, also produces and accumulates mannitol when cultivated in media containing high glucose concentrations [Groleau *et al*., 1995]. Similarly, *P. (Hansenula) anomala*, depending on the solute used to induce osmotic stress, also accumulates mannitol, in addition to glycerol, arabinitol and erythritol, in response to osmotic stress [Parekh and Pandey, 1985; van Eck *et al*., 1989]. Furthermore, van Eck and collaborators (1993) reported in a study using several yeasts, that *P. sorbitophila*, *C. versatilis*, *Candida cacoi*, *Zygosaccharomyces bisporus* and *Candida magnoliae* accumulate glycerol, arabinitol and mannitol, although no specific function has been suggested for mannitol. In the case of the yeasts *Candida amylolentus* and *Sterigmatomyces halophilus,* it seems that trehalose, besides glycerol and mannitol are accumulated in response to a_w reduction [van Eck, 1988]. Certain osmotolerant yeasts, such as *Pichia misa*, grown in the presence of high glucose concentration (~30%) showed to produce, in addition to ethanol, a variety of polyhydric alcohols: glycerol, erythritol, D-arabitol and mannitol [Spencer and Sallans, 1956; Blakely and Spencer, 1962; Onishi and Suzuki, 1966]. These few results concerning mannitol accumulation in yeasts point to a role of this compound in the regulation of osmotic stress provoked by solutes other than salt. Curiously, *Torulopsis mannitofaciens* [Onishi and Suzuki, 1969], *Torulopsis versatilis* [Lodder and Kreger-van Rij, 1952], and *Torulopsis anomala* [Lodder and Kreger-van Rij, 1952], recently reclassified as *C. mannitofaciens* (the first) and *C. versatilis* (the second and third) [Barnett *et al*., 1990], were isolated, in a similar way as *C. halophila*, from soy-sauce mash and other similar media such as pickling brines, and are excellent mannitol producers. *T. mannitofaciens* was reported to produce exclusively mannitol from both glucose and glycerol, but *T. versatilis* and *T. anomala* were good mannitol producers when using a large range of simple carbon sources such as glucose, fructose, mannose, galactose, maltose, glycerol and xylitol [Onishi and Suzuki, 1968,1970]. Recently, these yeasts were reclassified [Barnett *et al*., 2000; Kurtzman and Fell, 1998] and grouped together in the *C. versatilis* species group. We may speculate that the provenience of these yeasts may account for similar physiological characteristics, in which mannitol production as well as the ability to grown at least up to 2.8M of NaCl, seem to be, for instance, very good representative features.

Several yeasts accumulate other polyols besides glycerol, although in most cases their true role unknown (Table 2.2). On the other hand, fungi belonging to species such as *Aspergillus* [Adler *et al*., 1982], *Geotrichum* [da Costa and Nobre, 1989; Luxo *et al*., 1993], *Endomyces* [da Costa and Nobre, 1989; Luxo *et al*., 1993] and *Penicillium* [Adler *et al*., 1982], seem to have a preference for mannitol and arabinitol for osmoregulation purposes (Table 2.2), however the reason for this preference remains to be unveiled. Apparently, polyol accumulation seems to be related with each type of microorganism metabolism and also with the environment from which they were first isolated and in which they were naturally acclimated [Onishi, 1960] as well as the carbon source available for growth [da Costa and Nobre, 1989].

Furthermore, being compatible solute appears to be a specialized function of a compound also produced by an organism for other reasons [Blomberg and Adler, 1992]. Hence, it would be at first hand expectable that other compounds besides glycerol could as well perform the compatible solute function (Table 2.2).

Table 2.2. Examples of polyols accumulated by several yeasts, filamentous fungi and unicellular algae, as a response to the presence of NaCl in the medium. Polyols in bold have been published as salt stress responsive.

Organism	Polyols accumulated	References
Algae		
Dendryphiella salina	**Mannitol**, arabinitol, glycerol and erythritol	Jennings and Burke, 1990
Dunaliella spp	**Glycerol**	Brown, 1976
Black merismatic fungi		
Hortaea werneckii	**Glycerol**	Sterflinger, 1998; Petrovic *et al*., 2002
Cladosporium sphaerospermum	**Glycerol**	Sterflinger, 1998
Arxula adeninivorans	**Glycerol**	Yang *et al*., 2000
Filamentous fungi		
Penicillium chrysogenum	**Mannitol, glycerol**, arabinitol, erythritol	Adler *et al*., 1982
Aspergillus niger	**Mannitol**, **glycerol,** arabinitol, erythritol,	Adler *et al*., 1982
Geotrichum and Endomyces spp	**Arabinitol**, mannitol	da Costa and Niederpruem, 1980, 1982; Luxo *et al*., 1993
Yeasts		
Candida cacaoi	**Glycerol**, arabinitol,	van Eck *et al*., 1993
Candida glycerinogenes	**Glycerol,** arabitol	Zhuge *et al*., 2001
Candida magnoliae	**Glycerol, mannitol**	van Eck *et al*., 1993
Candida sake	**Glycerol,** arabitol, mannitol,erythritol	Abadias *et al*, 2001
Candida tropicalis	**Glycerol**	García *et al*., 1997
Cryptococcus amylolentus	**Glycerol, mannitol, trehalose**	van Eck, 1988
Debaryomes hansenii	**Glycerol**, arabinitol, ribitol,	Gustafsson and Norkrans, 1976; Adler and Gustafsson, 1980 ; André *et al*., 1988; Nobre and da Costa, 1985a,b; da Costa and Nobre, 1989; Larsson *et al*., 1990; Meikle *et al*., 1991
Pichia anomala	**Glycerol**, arabinitol, erythritol, mannitol	Parekh and Pandey, 1985; van Eck *et al*., 1989
Pichia farinosa	**Glycerol**, arabinitol, erythritol	Da Costa and Nobre, 1989; Höötmann *et al*., 1991
Pichia sorbitophila	**Glycerol**, arabinitol	van Eck *et al*., 1993
Saccharomyces cerevisiae	**Glycerol**	Edgley and Brown, 1978; Ölz *et al*., 1993
Sterigmatomyces halophiles	**Glycerol, mannitol, trehalose**	van Eck, 1988
Yarrowia lipolytica	**Glycerol**	Andreishcheva *et al*., 1999
Zygosaccharomyces bisporus	**Glycerol**, arabinitol,	van Eck *et al*., 1993
Zygosaccharomyces rouxii	**Glycerol, arabinitol**, mannitol	van Zyl *et al*., 1990; Groleau *et al*., 1995

2.1.3. *Meaning of trehalose and acetate production in yeasts*

Trehalose

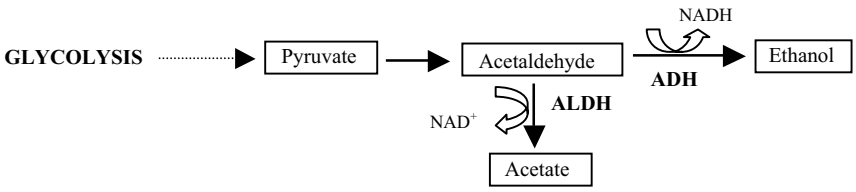
Considerable evidence over the past few years indicates that the disaccharide trehalose is widely distributed in nature and can be found in many organisms, including bacteria, fungi, plants, invertebrates and mammals. Generally trehalose has been shown to be used as a potential carbon and energy source, a reserve carbohydrate and a protective metabolite able to counteract deleterious effects of environmental stresses, due to its particular physical features [Argüelles, 2000]. Many yeasts accumulate trehalose in response to nutrient starvation and environmental stresses, and break it down when conditions favouring growth are restored. However yeast cells are largely unable to grow on trehalose as carbon source [Barnett *et al*., 2000]. In these lower eukaryotes, trehalose appears to play a dual function: as a reserve compound, mainly stored in vegetative resting cells and reproductive structures, and as stress metabolite [van Laere, 1989; Argüelles, 2000]. Yeast cells with higher intracellular concentrations of the reserve carbohydrate trehalose, are tolerant to adverse environmental conditions such as heat, desiccation, freeze-drying, hyperosmotic shocks and other, meaning that it may protect yeasts from stress-induced cellular damage [Gadd *et al*., 1987; Majara *et al*., 1996; Sharma, 1997; Parrou *et al*., 1997; Sano *et al*., 1999; Benaroudj *et al*., 2001]. Rapid mobilization of this reserve carbohydrate is associated with growth resumption, suggesting that its energy supply function may be a critical factor in overcoming nutritional imbalance during stress [Cansado *et al*., 1998]. Some authors reported that trehalose is degraded markedly even when trehalose accumulation is obvious, functioning as a futile cycle [Hottiger *et al*., 1987; Winkler *et al*., 1991]. Transient accumulation of trehalose following a hyperosmotic salt shock has been reported in *S. cerevisiae* [Gadd *et al*., 1987; Mackenzie *et al*., 1988; Meikle *et al*., 1988; Singh and Norton 1991; Hounsa *et al*., 1998; Oliveira and Lucas, 2004] and in *Torulaspora delbrueckii* [Nakata *et al*., 1995], though apparently with no straightforward relation with the response to salt stress. Studies made with the objective of unveil the *action mode* of trehalose in cells submitted to high levels of dehydration, revealed an effect at the cellular membrane's [Crowe *et al*., 1984]. Since high salt concentrations affect the membrane's composition [Watanabe and Takakuwa, 1984, 1987; Combs *et al*., 1968; Tunblad-Johansson *et al*., 1987; Tunblad-Johansson and Adler, 1987; Khaware *et al*., 1995; Petrovic *et al*., 1999; Turk *et al*., 2004], it is likely that trehalose accumulation may act as a stabilizer of the cellular membranes in several stress conditions, including salt stress [Crowe *et al*., 1984; Sharma, 1997]. Nevertheless, the role of trehalose as an osmoprotectant in yeasts is probably less important than glycerol´s [Thevelein, 1996]. As said above trehalose may result from a futile cycle, in which the major function seems to be the control of glucose influx into glycolysis, and simultaneously the recover of the inorganic phosphate pool [Thevelein and Hohmann, 1995]. These are eventually the most important roles underlying trehalose accumulation in yeasts.

The accumulation of trehalose has been demonstrated to be a crucial factor implied in the adaptive response to a variety of stresses, namely those induced by nutrient starvation, heat shock, dehydration or oxidative agents [van Laere, 1989; Majara *et al*., 1996; Benaroudj *et al*., 2001]. The enzymes required for both trehalose synthesis and hydrolysis in yeasts behave as general stress-responsive proteins [Winderickx *et al*., 1996]. Thus, trehalose seems to make part of the basic machinery of the cell response to environment changes and may help to understand the overlapping resistance between salt, ethanol, temperature, hydrogen peroxide and other, found in *S. cerevisiae* [Sharma, 1997; Hohmann, 1997; Gasch *et al*., 2000; Causton *et al*., 2001].

Acetate

Acetic acid is a normal by-product of the alcoholic fermentation processed by *S. cerevisiae*. Its production is strain dependent and favoured by growth at alkaline pH values [Radler, 1993]. Ethanol fermentation is a neutral-redox process, and thus the enhanced need for NAD(P)H during growth may be accounted by an increase in acetic acid production, since for every mole of acetate produced, 2 moles of NAD(P)H will be available for further reduction [Brown and Edgley, 1980]. During alcoholic fermentation excess of NADH generated during biomass formation is usually spent by the formation of glycerol [Albers *et al*., 1996; van Dijken and Scheffers, 1986]. Thus, acetate accumulation seems to appear as an extra necessity of NAD(P)H re-oxidation. In fact, Blomberg and Adler (1989) have reported several years ago, in *S. cerevisiae,* that the presence of 0.7M NaCl led to an increase in the amount of acetate produced by the cells. According to these authors, the enhanced need for NADH during the conditioning in 0.7M NaCl may be partly accounted for by an increased rate in acetate production. Moreover, the enhanced rate of acetate production is reflected in changed activities of the two enzymes competing for acetaldehyde as substrate: the metabolic flow to acetate is favoured by a reduction in ADH activity and an increment in ALDH activity (Figure 2.1).

Figure 2.1. Glycolysis branch for ethanol and acetate production. ADH-Alcohol dehydrogenase; ALDH-acetaldehyde dehydrogenase.



Similarly to what was found for trehalose accumulation, *S. cerevisiae* acetate production in chemostat cultures increased with increasing dilution rates, *i.e.*, in the cases in which fermentative metabolism became predominant [Ölz *et al.*, 1993]. At higher dilution rates, in *S. cerevisiae*, which may be somehow related with the increase in acetate production. As stated before by Blomberg and Adler (1989), Ölz and collaborators (1993) reported also an increase in acetate production under salt stress conditions, maintaining the same relation with dilution rates as in the absence of salt. On the other hand, Remize and collaborators (1999, 2000), verified that an attempt to overproduce glycerol in wine making *S. cerevisiae* industrial strains led to an increase in acetate production, which is undesirable in wine and beer production. The marked increase in glycerol production led to a more pronounced deficit in reduced cofactors, and therefore acetate production results as a mean of generating reduced cofactors. Following this reasoning, it will be expected that enhanced glycerol production and accumulation under salt stress, lead to an increase in acetate production.

Brettanomyces and *Dekkera* species are well known for their intensive production of acetic acid from glucose under aerobic conditions, which if not neutralized by the addition of calcium carbonate to the medium leads to their death [Pitt and Hocking, 1997]. Secretion of acetate cause in these yeasts a lack of NAD^+, which alters the NAD^+/NADH ratios resulting in an unfavoured redox balance [Walker, 1998]. Apparently intracellular reoxidation of NADH via other routes, such as glycerol

production, is too low. In fact, in *Brettanomyces intermedius* it was not observed glycerol production, which may be explained by the absence of glycerol-3-phosphate dehydrogenase activity [Wijsman *et al*., 1984]. When cells of *B. intermedius* are exposed to small amounts of oxygen or organic hydrogen acceptors, *e.g*., acetoin, a stimulation of ethanol production is verified (known as Custers effect), which aims the recover of redox balance through the generation of NAD^+ at expense of ethanol production [Wijsman *et al*., 1984] (Figure 2.1.). The metabolic limitation concerning NADH re-oxidation through glycerol production in *Brettanomyces* and *Dekkera* species might explain their lower resistance to low a_w provoked by NaCl [van Eck *et al*., 1993; Lages *et al*., 1999].

2.1.4. *Growth and assimilation tests pattern of* C. halophila

According to Barnett and collaborators (1990) and as it can be seen in Figure 2.2, *C. halophila* does not use a high number of different compounds as carbon and/or energy sources.

Figure 2.2. Description of *Candida halophila* species according to Barnett and collaborators (1990).

CANDIDA HALOPHILA
Yarrow and Meyer (1978)

Synonyms *Torulopsis halophilus* Onishi (nom. nud)

Description white; butyrous colonies; vegetative reproduction by budding; no filaments; no sexual reproduction

Fermentation

F1 D-Glucose +	F4 Me α-D-Glucoside –	F7 Melibiose –	F11 Raffinose –
F2 D-Galactose D	F5 Sucrose D	F8 Lactose –,D	F12 Inulin –
F3 Maltose –	F6 α,α-Trehalose –,D	F9 Cellobiose D	F13 Starch –
		F10 Melezitose –	F14 D-Xylose –

Growth

C1 D-Glucose +	C20 Melezitose –	C39 Succinate +	V4 w/o Biotin –
C2 D-Galactose +	C21 Inulin –	C40 Citrate +	V5 w/o Thiamin –
C3 L-Sorbose –	C22 Starch –	C41 Methanol –	V6 w/o Biotin and Thiamin –
C4 D-Glucosamine –	C23 Glycerol +	C42 Ethanol +	V7 w/o Pyridoxine +
C5 D-Ribose –	C24 Erythritol –	C43 Propane 1,2 diol –	V8 w/o Pyridoxine and Thiamin –
C6 D-Xylose –	C25 Ribitol –	C44 Butane 2,3 diol –	V9 w/o Niacin +
C7 L-Arabinose –,D	C26 Xylitol D	N1 Nitrate +	T1 at 25ºC +
C8 D-Arabinose –	C27 L-Arabinitol –	N2 Nitrite +	T2 at 30ºC +
C9 L-Rhamnose –	C28 D-Glucitol –	N3 Ethylamine +	T3 at 35ºC –
C10 Sucrose D	C29 D-Mannitol +	N4 L-Lysine +	T4 at 37ºC –
C11 Maltose –	C30 Galactitol –	N5 Cadaverine +	T5 at 40ºC –
C12 α,α-Trehalose +	C31 *myo*-Inositol –	N6 Creatine +	O1 0.01% Cycloheximide –
C13 Me α-D-Glucoside –	C32 D-Glucono-1.5- lactone –	N7 Creatinine –	O2 0.1% Cycloheximide –
C14 Cellobiose +	C33 5-Keto-D-gluconate +	N8 Glucosamine –	O3 1% Acetic acid –
C15 Salicin +	C34 5-Keto-D-gluconate ?	N9 Imidazole –	O4 50% D-Glucose +
C16 Arbutin +	C35 D-Gluconate –	V1 w/o vitamins –	O5 60% D-glucose +
C17 Melibiose –	C36 D-Glucuronate –	V1 w/o vitamins –	
C18 Lactose –,D	C37 D-Galacturonate –	V2 w/o *myo*-Inositol +	
C19 Raffinose –	C38 DL-Lactate –	V3 w/o Pantothenate +	

Additional characteristics

M1 Starch formation –	M2 Acetic acid production –	M3 Urea hydrolysis –	M4 Diazomium Blue B reaction –

Using classical assimilation assays *C. halophila* showed to be able to assimilate glucose (either fermenting or respiring), galactose, α,α-trehalose, cellobiose, salicin, arbutin, glycerol, D-mannitol, 2-keto-D-gluconate, succinate, citrate and ethanol as carbon and energy sources (Figure 2.2). This selectivity is most probably related with the environment of origin (soy mash) and the fermentation process of soy sauce production in which it is used, reason why it is termed a soy-sauce yeast [van der Sluis *et al*., 2000; 2001]. Soy-sauce production is a fermentation process mainly performed by bacteria, which deplete most of the available sugars and simultaneously produce lactate, acetate and

ethanol [Röling *et al*, 1994a,b]. After this fermentation takes place and due to the acids production, the medium pH drops to almost 4.5 and bacteria can not grow anymore. On contrary, this pH allows yeasts to grow, but then the available compounds as carbon sources are the sub-products of bacteria metabolism, namely acids. For this reason, and taking into consideration that soy-sauce production process takes place in the presence of about 16 to 18% (~ 3M) of salt [Röling *et al*, 1994 a,b], only some yeasts and with limited number of duplications will be able to proceed with fermentation. In fact, the flavour development in the soy-sauce process normally takes a long time because the metabolic activity of the salt-tolerant yeasts is low not only due to the high salt content of the soy-sauce medium but also due to the slurry-state of the soy-sauce medium, which makes the substrates poorly available for the yeasts [van der Sluis *et al*., 2001]. However, such high salt content seems to be beneficial for the production of the soy-sauce flavour. Since *C. halophila* is a soy-sauce yeast isolated from a soy-sauce component, the use of limited range of substrates seems to be reasonably explained.

Recently, Kurtzman and Fell (1998) and Barnett and collaborators (2000) reclassified the yeast *C. halophila* into *C. versatilis* (Figure 2.3.).

Figure 2.3. Description of *Candida versatilis* species according to Barnett and collaborators (2000).

Candida versatilis
(Etchells and T.A. Bell) S.A. Meyer and Yarrow (Yarrow and Meyer 1978)

Synonyms
Brettanomyces versatilis Etchells and T.A. Bell; *Candida halophila* Yarrow and S.A. Meyer;
Candida mannitofaciens (Onishi and Suzuki) S.A. Meyer and Yarrow; *Candida taiwanensis* F.-L. Lee *et al.* ;
Debaryomyces tamarii Y. Ohara and Nonomura *ex* van der Walt and E. Johannsen;
Debaryozyma tamarii (Y. Ohara and Nonomura *ex* van der Walt and E. Johannsen) van der Walt and E. Johannsen;
Pichia tamarii (van der Walt and E. Johannsen) Campbell;
Torulaspora tamarii (Y. Ohara andNonomura ex van der Walt and E. Johannsen) van der Walt and E. Johannsen;
Torulopsis anomala Lodder and Kreger-van Rij;
Torulopsis halophilus Onishi ; *Torulopsis mannitofaciens* Onishi and T. Suzuki;
Torulopsis versatilis (Etchells and T.A. Bell) Lodder and Kreger-van Rij

Description white to cream; butyrous colonies; vegetative reproduction by budding; no filaments; no sexual reproduction

Fermentation

F1 D-Glucose +,D	F4 Me α-D-Glucoside –	F7 Melibiose D, –	F11 Raffinose D,–
F2 D-Galactose +,D	F5 Sucrose +,D	F8 Lactose +,–	F12 Inulin –
F3 Maltose +,–	F6 α,α-Trehalose +,–	F9 Cellobiose +,D	F13 Starch –
		F10 Melezitose –	F14 D-Xylose –

Growth

C1 D-Glucose +	C21 Inulin –	C41 Methanol –	V6 w/o Biotin and Thiamin –
C2 D-Galactose +	C22 Starch –	C42 Ethanol +,D	V7 w/o Pyridoxine +
C3 L-Sorbose –	C23 Glycerol +	C43 Propane 1,2 diol –	V8 w/o Pyridoxine and Thiamin –
C4 D-Glucosamine –	C24 Erythritol –	C44 Butane 2,3 diol D,–	V9 w/o Niacin +
C5 D-Ribose +,–	C25 Ribitol D,–	N1 Nitrate +	V10 w/o PABA +
C6 D-Xylose D,–	C26 Xylitol D,-	N2 Nitrite +	T1 at 25ºC +
C7 L-Arabinose +,–,D	C27 L-Arabinitol D,–	N3 Ethylamine +	T2 at 30ºC +,w
C8 D-Arabinose –	C28 D-Glucitol –	N4 L-Lysine +	T3 at 35ºC +,–
C9 L-Rhamnose –	C29 D-Mannitol +,-	N5 Cadaverine +	T4 at 37ºC –
C10 Sucrose +,–	C30 Galactitol –	N6 Creatine +,–	T5 at 40ºC –
C11 Maltose +,–	C31 *myo*-Inositol –	N7 Creatinine –	T6 at 42ºC –
C12 α,α-Trehalose +,–	C32 D-Glucono-1.5-lactone +,–	N8 Glucosamine –	T7 at 45ºC –
C13 Me α-D-Glucoside –	C33 2-Keto-D-gluconate +,–	N9 Imidazole –	O1 0.01% Cycloheximide +, –
C14 Cellobiose +	C34 5-Keto-D-gluconate –	N10 D-Tryptophan –	O2 0.1% Cycloheximide +, –
C15 Salicin +,–	C35 D-Gluconate D,–	V1 w/o vitamins –	O3 1% Acetic acid –
C16 Arbutin +,D	C36 D-Glucuronate –	V2 w/o *myo*-Inositol +	O4 50% D-Glucose +
C17 Melibiose +,–	C37 D-Galacturonate –	V3 w/o Pantothenate +	O5 60% D-Glucose +
C18 Lactose +,–	C38 DL-Lactate w,–	V4 w/o Biotin –	O6 10% NaCl +
C19 Raffinose +,–	C39 Succinate +,–	V5 w/o Thiamin –	O7 16% NaCl +
C20 Melezitose –	C40 Citrate +,–		

Additional characteristics

M1 Starch formation –	M2 Acetic acid production –	M3 Urea hydrolysis –	M4 Diazomium Blue B reaction –

If we compare the data in Figure 2.2. and 2.3., we do not retain anymore the impression of the carbon source use limitation that *C. halophila* indeed has, since some of the yeasts belonging to this species group can use a wider range of compounds as carbon and/or energy sources (shaded cells). This type of classification tests can be misleading as to the true metabolic abilities of a certain yeast strain. In fact, *C. halophila* showed to behave, in terms of salt resistance, very differently from *C. versatilis* type strain, being less salt resistant in the survey performed by Lages and collaborators (1999). Thus we considered that metabolic differences between strains of the same species were sufficiently important for keeping *C. halophila* designation throughout this thesis. Although this assumption may be contestable, it has been recently emphasized by Oren (2002), who stated that microbiologists were not aware of the true extent of the diversity of halophilic and halotolerant microorganisms in nature, being the diversity expressed both the phylogenetic level and at the physiological metabolic level.

2.2. Results and discussion

2.2.1. *Growth studies in single carbon sources*

We initiated the physiological characterization of *C. halophila* growth with the determination of growth curves at 30ºC, in erlenmyer batch systems using two different media, yeast extract peptone dextrose (YEPD) and mineral medium (MM), with 2% (w/v) of glucose as carbon and energy source. These media were tested at initial pH´s ranging from 3 to 7. *C. halophila* cells growing in YEPD presented a higher specific growth rate than in MM, though not varying significantly within the values of initial medium pH assayed (Table 2.3). Dry weight determinations showed significant differences between those two media. While cells cultivated in MM have their final dry weight increased with increasing initial medium pH, YEPD-grown cells showed a reduction in their final biomass with increasing initial medium pH. However, the maximum value of dry weight obtained using MM neither surpasses nor equals the minimum value of dry weight obtained in YEPD-grown cells.

Table 2.3. Growth kinetic parameters of *C. halophila* cells grown in YEPD and MM at 30ºC and 160 r.p.m. at different initial medium pHs. Dry weight was measured in stationary phase.

	YEPD		MM	
Initial pH	μ_g (h^{-1})	**Dry weight** ($g\ l^{-1}$)	μ_g (h^{-1})	**Dry weight** ($g\ l^{-1}$)
3	--*	--	0.100 ± 0.007	1.92 ± 0.37
4	0.168	9.16 ± 0.14	0.116 ± 0.010	2.77 ± 0.24
5	0.177	7.62 ± 0.04	0.107 ± 0.028	2.94 ± 0.34
6	0.172	7.56 ± 0.05	0.102 ± 0.016	4.50 ± 1.03
7	0.161	5.98 ± 0.01	n.d.	n.d.

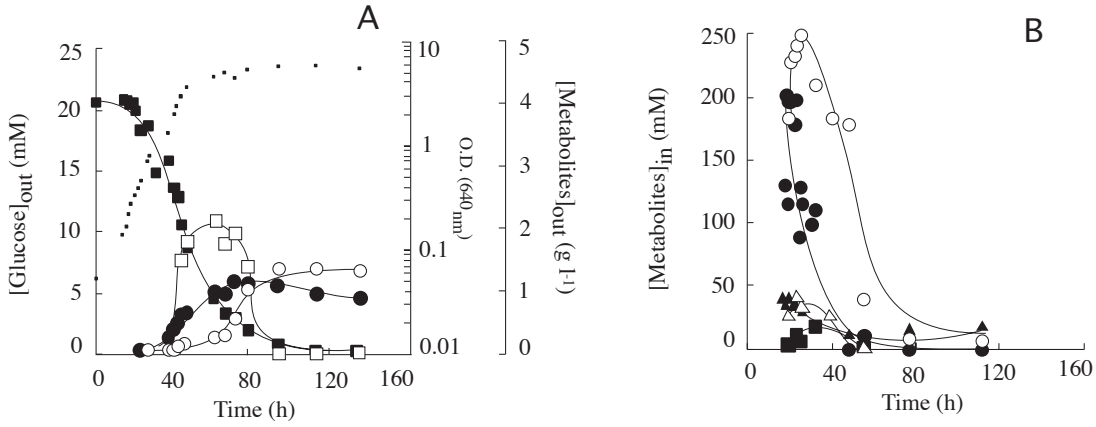
*The composition of YEPD does not allow this pH value; n.d. not determined

Since YEPD is a complex medium possessing several compounds that can be used as carbon sources and other that may be used directly and do not need to be synthesized by the cell as in MM, it is not surprising the results concerning the final biomass, *i.e.*, higher final dry weight values (Table 2.3).

On the other hand, and considering the medium composition, the relation of YEPD medium with initial medium pH have their interpretation, most probably, linked with their lower buffer capacity at higher pH values and thus, a reduction in the extracellular pH caused by metabolism is faster at these pH values, not allowing the cells to proliferate, and consequently lowering the dry weight values. We decided to use throughout the work MM, because it is a well defined medium which full composition is known and which allows us a comparison of the metabolism with *S. cerevisiae*, in which most information on main metabolic fluxes under glucose repression have been well studied. Temperature influence in specific growth rate was also studied and since it did not vary significantly from 25ºC to 30ºC (not shown), all the assays performed in the scope of this thesis were made at 30ºC.

Yeasts growing actively at the expense of a certain carbon source produce and excrete several secondary products. Thus, glucose growing cultures of *C. halophila* were monitored through the simultaneous measurement of the optical density and by the determination of the amounts of the compounds present both in the medium and intracellularly. *C. halophila* growing on glucose containing medium, produces and accumulates during exponential phase, mannitol, glycerol, trehalose, and ethanol (Figure 2.4). If we compare the graphs A and B its clear that the extracellular increasing of mannitol and glycerol concentrations accompany the decrease of their intracellular concentrations, showing that these compounds accumulate transiently inside the cell, being further excreted to the medium.

Figure 2.4. Growth -O.D. (▪), consumption of glucose (■) and accumulation of acetate (△), ethanol (□), glycerol (●), mannitol (○) and trehalose (▲) **A-** Extracellularly and **B-** Intracellularly, in batch cultures of *C. halophila* grown in MM at 30ºC supplemented with 2%(w/v) of glucose.



Acetate, trehalose and glucose were also measured intracellularly especially in the begining of the exponential phase, and although their concentrations were quite low, they slightly decreased during exponential phase to values near of zero, with exception of trehalose that maintained a concentration of about 10 mM (Figure 2.4).

As said before, *C. halophila* is able to use mannitol and glycerol as carbon sources. Besides,

as a soy-sauce yeast, it is likely that it may grow as well with ethanol as carbon source. The kinetic parameters of *C. halophila* cells grown on these substrates are presented in Table 2.4. As can be seen, glycerol and mannitol are used at the same growth rate, which, in turn is also similar to the growth rate obtained with glucose as carbon source (Table 2.3).

Table 2.4. Growth parameters of *C. halophila* cells grown in MM at pH 5.0, supplemented with 2% (w/v) of different carbon sources at 30ºC and 160 r.p.m.. Dry weight measured in the stationary phase.

Carbon source	μ_g (h^{-1})	**Dry weight** (g l^{-1})
Glycerol	0.103 ± 0.005	3.93 ± 0.39
Mannitol	0.094 ± 0.008	4.46 ± 0.45
Ethanol	0.039 ± 0.003	4.76 ± 0.28

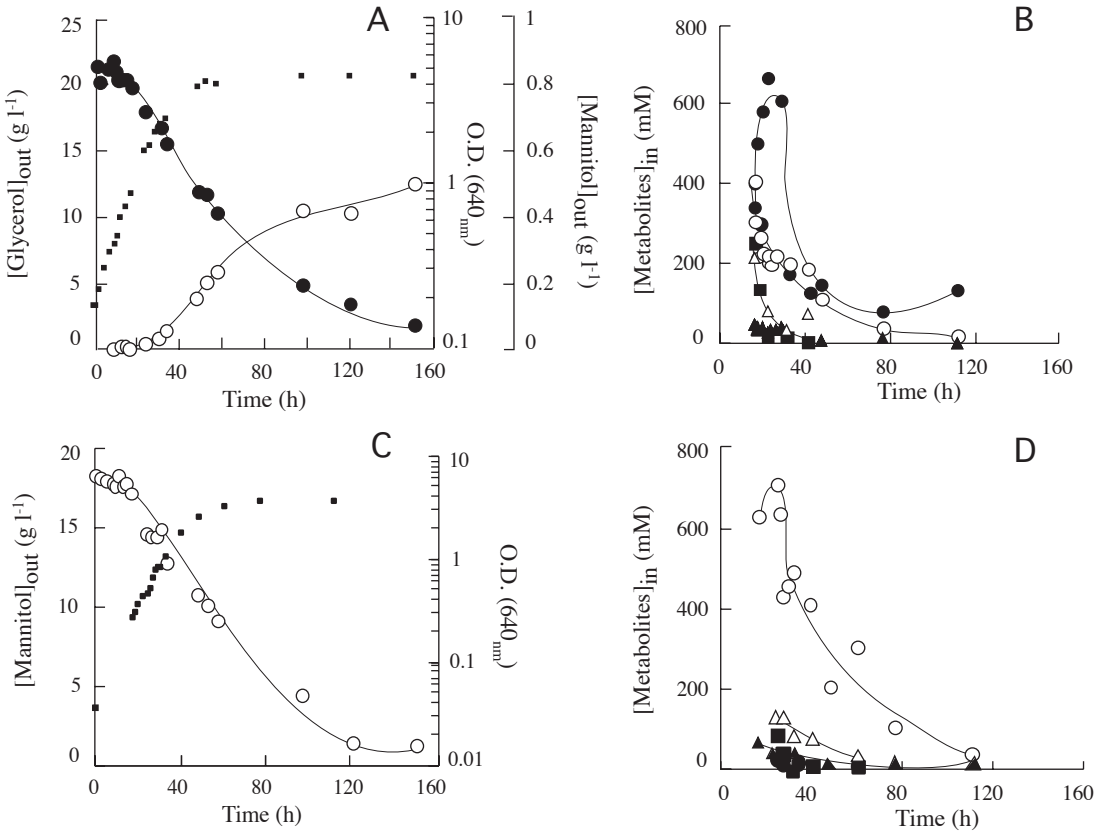
C. halophila was able to use ethanol as carbon source, with a specific growth rate about 38% of the value of the specific growth rate of glucose grown cells. This value is very similar to the one found in *S. cerevisiae* in identical culture conditions, in which a reduction of 41% in the specific growth rate was observed [Lages and Lucas, 1997]. *S. cerevisiae* is widely known for their fermentative ability, being thus quite *familiar* with the presence of ethanol, nevertheless is has a percentage of reduction in specific growth rate similar to *C. halophila*. Since *C. halophila* was isolated from soy mash and participates in the last stage of the soy-sauce fermentation process it may be adapted as well as *S. cerevisiae* to the presence of ethanol.

Biomass achieved in stationary phase does not accompany the variations observed in growth rates. Comparing growth at pH 5, one realises that *C. halophila* is able to produce ≈ 60% more biomass on ethanol than in glucose-grown cells (Table 2.3 and 2.4). This value is identical for biomass produced in glucose-grown cells at pH6, which stresses the pH dependence of carbon source consumption mentioned in the introduction, but may also be a consequence of the soy-sauce process from which *C. halophila* was isolated. In spite of the reduced growth rate, ethanol grown cells achieved the highest final dry weight of all the carbon sources (Table 2.4).

Considering that glycerol and mannitol were detected as the main metabolites accumulated in glucose grown cells and can be used at identical growth rates as carbon sources, we decided to perform similar studies as those in Figure 2.4, but with mannitol and glycerol as carbon sources (Figure 2.5). As in glucose grown cells, mannitol and glycerol appeared as the main metabolites accumulated and excreted by *C. halophila* growing exponentially in medium with glycerol as carbon source (Figure 2.5 A and B). Mannitol was synthesized from the beginning of the exponential phase and then continuously delivered into the medium till the exhaustion of the carbon source. Similarly to what was reported in glucose grown cells, acetate and trehalose and glucose to a much lower extent, were also detected in the begin of the exponential phase and then reduced, being their values almost null at the end of the exponential phase. Although trehalose registered some variation during growth, its concentration during exponential and stationary phase was identical to glucose grown cells, around 10 mM. Mannitol grown cells showed very low intracellular amounts of glycerol (Figure 2.5. C and D). This compound was detected just in the beginning of the exponential and concentrations did not surpass 10

mM. On the other hand, intracellular mannitol concentration increased slightly and then decreased continuously till the complete exhaustion of the extracellular mannitol concentration. Mannitol accumulated intracellularly may be the result of the accumulation of extracellular mannitol inside the cell due to the action of a possible mannitol transporter at a higher rate than mannitol consumption pathways are able to use it. Acetate, glucose and trehalose intracellular concentrations did not differ significantly from glycerol-grown cells (Figure 2.5 B and D).

Figure 2.5. Growth (O.D.), glucose consumption and production of the metabolites in glycerol grown cells (**A** and **B**) and mannitol grown cells (**C** and **D**), in batch cultures of *C. halophila* grown in MM at 30ºC supplemented with 2%(w/v) of each carbon source. Symbols: O.D. (▪),acetate (△), glucose (■), glycerol (●), mannitol (○), trehalose (▲).



C. halophila cells growing on ethanol as carbon source were also assayed for their intracellular production of metabolites, however not so exhaustively as the other carbon sources (not shown). Once more, mannitol and trehalose were detected intracellularly and gradually decreased with the progression of the exponential phase (not shown). As in mannitol grown cells no glycerol was detected either intracellularly or extracellularly (not shown). As expected and according to the membrane protector characteristics of trehalose, their intracellular values were higher than in all the

other carbon sources tested (not shown).

The metabolic roles of the intracellular solutes can be discussed on the light of what is known in other yeasts, in particular *S. cerevisiae* (Section 2.1.2). Glycerol might be produced for redox balance purposes, like presented in section 2.1.2. Nevertheless, we cannot exclude the possibility that mannitol might instead be fulfilling those needs in *C. halophila*, since the amounts of mannitol are generally higher than glycerol´s and it is found in cells growing in all carbon sources. This issue will be further addressed in chapter 4. We can, however not disregard the multiplicity of roles and different cases described in the literature (Section 2.1.2), reason why it is not clear which would be the physiological role played by mannitol in *C. halophila*. The hypothesis of it being compatible solute will be addressed further ahead.

The ubiquitous presence of small amounts of trehalose appears to be related to growth phase regardless of the carbon source. Trehalose has been proposed as regulator of the carbon source influx into glycolysis in *S. cerevisiae* [Thevelein and Hohmann, 1995], and thus it may be fulfilling the same role in *C. halophila*. In fact, the presence of intracellular glucose in glycerol, mannitol and ethanol-grown cells indicates that gluconeogenesis is functioning and trehalose might be exerting some kind of metabolic regulation.

Finally, acetate appears as the natural consequence of *C. halophila* metabolism accumulating intracellularly only in the exponential phase and decreasing steeply through the time. In *S. cerevisiae* acetate production increases slightly up to 50 hours of growth [Blomberg and Adler, 1989]. It should be noted that, although acetate may be produced for the same reasons in these two yeasts, they are, most probably, metabolically very different, and consequently, the production of acetate differs to some extent naturally.

2.2.2. *Growth studies in carbon source mixtures*

In nature microorganisms are faced constantly with a wide range of available compounds, which can be used as carbon or/and energy sources. For this reason, the characterization of growth in the presence of more than one carbon or/and energy source is of fundamental importance, not only to improve our basic concepts in microbial ecology, but also for a better understanding of, for example, the kinetics of pollutant degradation by microorganisms in the environment where such compounds *compete* with naturally available substrates [Egli *et al*., 1993]. There are essentially three modes of utilization of substrates mixtures: (i) simultaneous, in which two or more compounds are taken up and dissimilated simultaneously by the cell; (ii) sequential, which means that an hierarchy is established among the carbon sources available and only after the more or less complete exhaustion of the most easier assimilable carbon source, will be the other taken up by the cell and consequently used; (iii) diauxic, when the assimilation of the second carbon source occurs only after exhaustion of the first [Egli *et al*., 1993]. Mixed substrate growth is dependent on the type of substrates, their concentration and in the growth system used, being the consumption of substrates in batch systems different from those of chemostat cultures. Information available indicates that in batch cultures simultaneous utilization of substrates in dependent on low concentrations of initial substrates, and this must be strictly followed otherwise diauxic growth will be observed [Egli *et al*., 1993].

C. halophila was grown in mixtures of glucose, glycerol and mannitol in several combinations (Figure 2.6). As can be observed in Figure 2.6-A, glucose and glycerol are not utilized simultaneously by *C. halophila* cells, being glycerol consumed only after the glucose concentration

reached a level around 0.1-0.2%, indicating sequential consumption. On the other hand, glycerol and mannitol showed to be simultaneously consumed, although at different rates (Figure 2.6-B). If we observe carefully the Figure 2.6-B and C we notice that mannitol medium concentration increases slightly (about 1 mM) during lag phase and the beginning of exponential growth. This effect is most probably due to the production of mannitol by the cells with further excretion to the medium and this is only possible if we postulate that glycerol consumption begins earlier than mannitol´s. Though this is not straightforwardly visible in Figure 2.6, it is expectable according to results from glycerol consumption presented in Figure 2.5. Mannitol, similarly to what happen with glycerol, is not consumed simultaneously with glucose (Figure 2.6-C), but maintains the pattern of simultaneous consumption with glycerol after the exhaustion of glucose. Glucose is, as said above, the preferential substrate of yeasts, and when it is present in the medium triggers a series of reactions that lead to the regulation of the enzymes and the expression of genes involved in other metabolic pathways [Gancedo, 1992, 1998]. In yeasts the utilization of substrates is subjected to different regulation mechanisms, which, besides the underlying molecular mechanisms, may be distinguished from each other according to the time duration of the response to environment alterations.

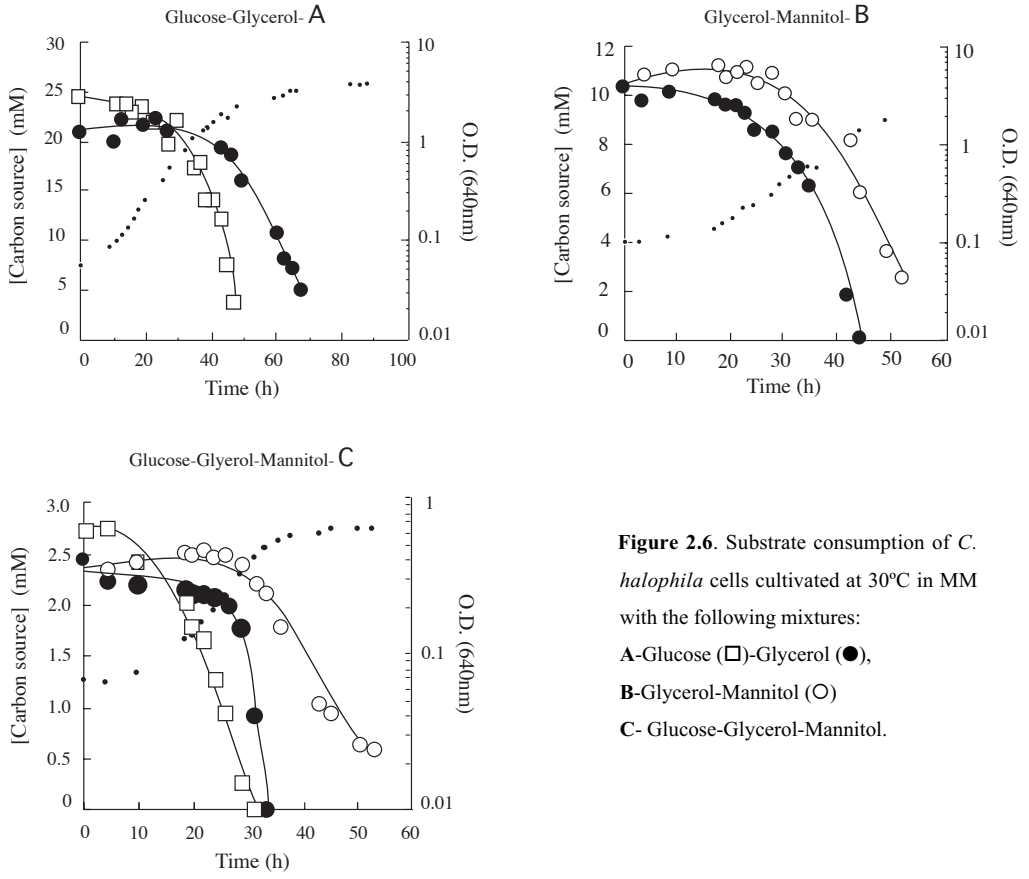


Figure 2.6. Substrate consumption of *C. halophila* cells cultivated at 30ºC in MM with the following mixtures:
A-Glucose (□)-Glycerol (●),
B-Glycerol-Mannitol (○)
C- Glucose-Glycerol-Mannitol.

While activation, desactivation and exchange are quick responses that may occur in seconds or few minutes, inactivation, induction and repression may take from several minutes to hours to occur. Regulation or repression by glucose is a well establish phenomenon among yeasts [Gancedo, 1992, 1998]. Nevertheless, information is scanty among the yeasts with respect to glucose, glycerol or mannitol mixtures consumptions. For example, the halotolerant yeast *P. sorbitophila*, which presents identical values for the growth rates of glucose and glycerol, consumes glucose and glycerol simultaneously [Lages and Lucas, 1995]. To our knowledge, there are only two cases of metabolic activity known to be subjected to the glucose effect in yeasts: glycerol utilization in *Candida valida* [Babel and Hofmann, 1982] and mannitol utilization in *C. albicans* [Niimi *et al.*, 1986]. *C. albicans* grown on a mixture of glucose and mannitol showed typical diauxic growth [Niimi *et al.*, 1986], in which mannitol utilization occurred only after exhaustion of glucose. Furthermore the activity of mannitol dehydrogenase, the first enzyme in mannitol utilization, was very low in the presence of glucose [Niimi *et al.*, 1986], and mannitol transport was detected on cells grown on mannitol [Niimi *et al.*, 1986], but not on glucose grown cells. These findings indicate that in *C. albicans* glucose represses the synthesis of mannitol dehydrogenase and mannitol transport system [Niimi *et al.*, 1986]. It remains to be known, in *C. halophila*, whether the regulation of the sequential growth observed has a counterpart at the level of substrate uptake and of enzymes responsible for their utilization, which will be reported in Chapter 3 and 4, respectively.

2.2.3. *Growth studies in the presence of salt*

C. halophila was reclassified as a strain of *C. versatilis*, and according to Barnett and collaborators (2000) (Figure 2.3) all the strains belonging to this group grow at least in the presence of 16% (w/v) NaCl (2.75M). Nevertheless, in the salt stress survey made by Lages and collaborators (1999), C. *halophila* revealed to be a halotolerant yeast able to grow at NaCl concentrations above 4M. Growth characterization begun by assaying *C. halophila* growth in MM containing increasing salt concentrations at an initial medium pH of 5.0. Growth was detectable up to 4.5M of NaCl with glucose and glycerol as carbon sources. In YEPD growth was only detected up to 3.5M. Growth curves of glycerol- and glucose–MM grown cells, in the presence of NaCl up to 3.5M were very similar, but, above this value growth was progressively reduced and the lag phase increased steeply (Figure 2.7). Similar growth curves have been presented through the years in other more or less halotolerant organisms such as the well known halotolerant yeast *D. hansenii* [Norkrans, 1966; Neves *et al.*, 1997], in *Z. rouxii* [Yagi and Nishi, 1993], in the yeasts *R. mucilaginosa*, *S. cerevisiae* and *P. guilliermondii* [Blomberg, 1997; Lahav *et al.*, 2002], in *A. adeninivorans* [Yang *et al.*, 2000] and in several xerophilic fungi [Pitt and Hocking, 1977]. Although presenting different levels of resistance to NaCl, all these organisms showed the same pattern of growth, *i.e.*, progressively slower growth with increasing salt concentrations concomitant with an extension of the respective lag phase duration.

Specific growth rates were calculated from the curves represented in Figure 2.7 and plotted as a function of the salt concentration in the medium (Figure 2.8-A). A set of results identical to the ones presented in Figure 2.7 for glucose and glycerol growing cells, using mannitol as single carbon source (not shown) was used to calculate growth parameters in Figure 2.8. As reported before for the halotolerant yeasts *D. hansenii* [Prista *et al.*, 1997] and *P. sorbitophila* [Lages, 2000], the specific growth rate of *C. halophila* increased 15 to 20% in the presence of salt up to 2M with a maximum at 1.5M. This behavior was observed in all the carbon sources assayed, indicating that this is an intrinsic characteristic of *C. halophila* cells, that seems to prefer a certain amount of salt, an aspect already

reported for this yeast by van der Sluis and collaborators (2001).

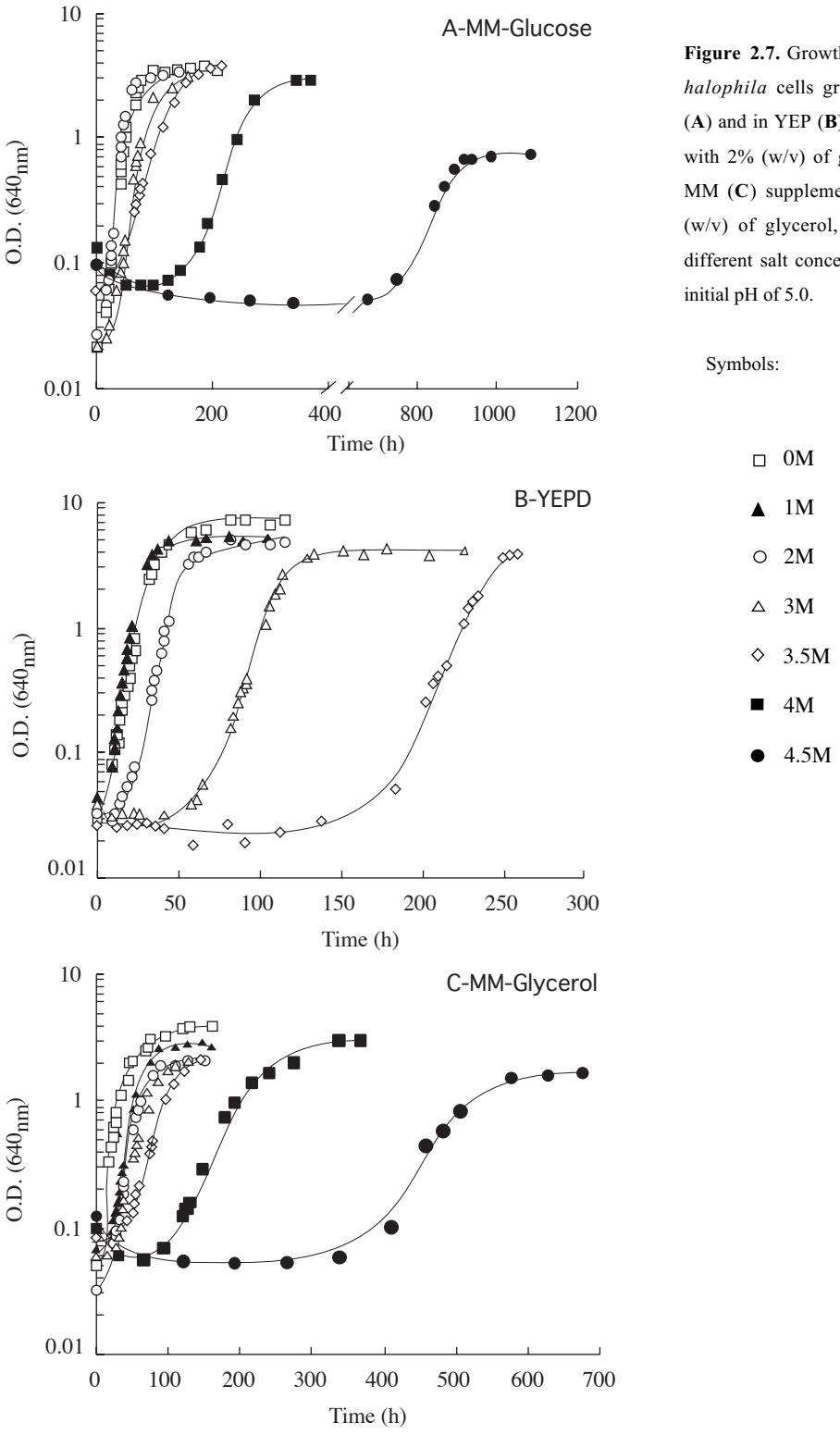


Figure 2.7. Growth curves of *C. halophila* cells growing in MM (**A**) and in YEP (**B**) supplemented with 2% (w/v) of glucose and in MM (**C**) supplemented with 2% (w/v) of glycerol, at 30ºC with different salt concentrations at an initial pH of 5.0.

Symbols:

In fact, the same effect has been reported in *P. sorbitophila* salt grown cells using glucose as carbon source, but not in cells using glycerol as carbon source [Lages, 2000]. *C. halophila* YEPD grown cells did not show an increase in their specific growth rate up to 2M as did MM grown cells (Figure 2.8-A). The hypothetical relation of this result with YEPD composition remains to be clarified.

Yeasts salt stress tolerance is dependent not only on the type of solute inducing stress, but also on the strain [Blomberg, 1997], the cells physiological state and growth conditions, namely temperature, nitrogen source, extracellular pH and media composition [Blomberg and Adler, 1989; Elliot and Futcher, 1993]. It is well known that actively growing cells are less resistant to salt than cells in stationary phase, a phenomenon that might have its interpretation in the osmolytes accumulated during stationary phase, which, in some yeasts differ substantially from those found in cells growing exponentially [Mackenzie *et al.*, 1986; Blomberg *et al*., 1988; Larsson and Gustafsson, 1993; Hounsa *et al*., 1998]. The importance of nitrogen source in salt tolerance of the halotolerant yeast *D. hansenii* was reported by Larsson and Gustafsson (1993), which observed a significant increase in salt tolerance level when the nitrogen source was changed from ammonium to urea.

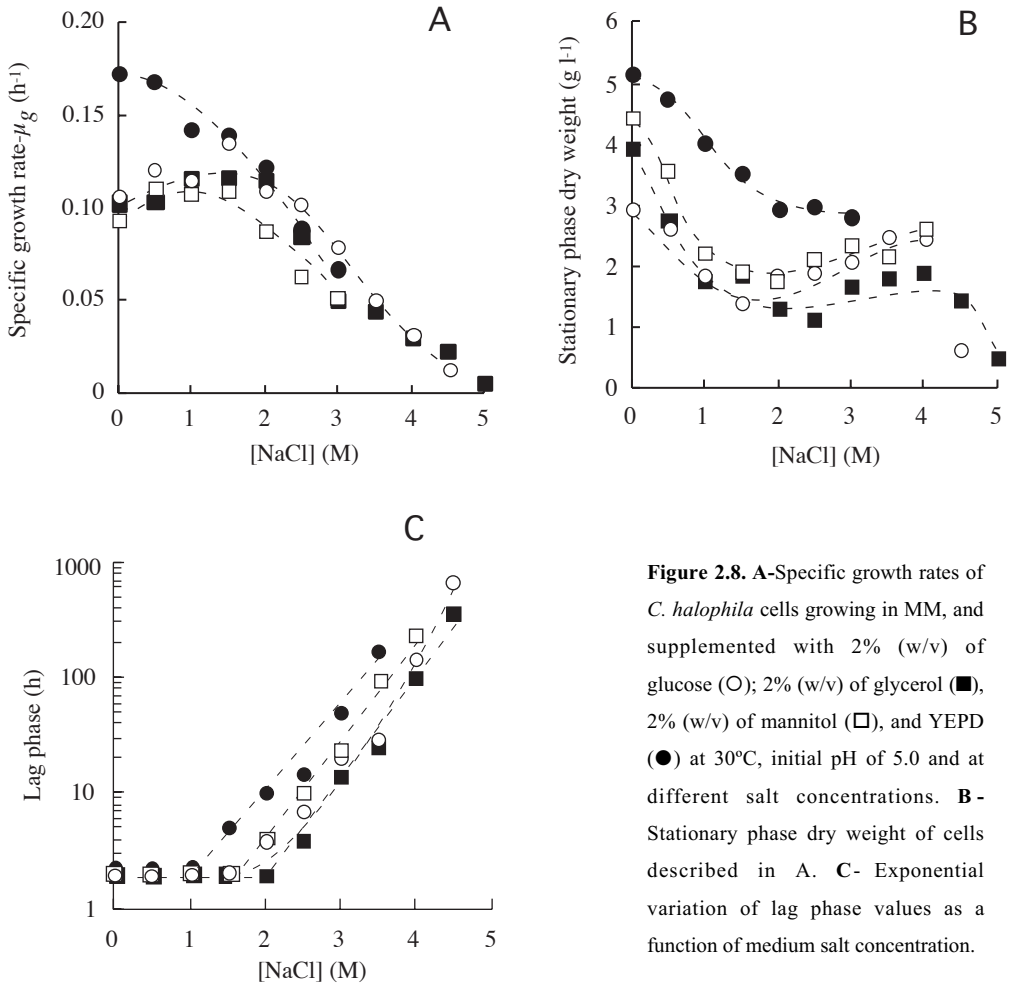


Figure 2.8. A-Specific growth rates of *C. halophila* cells growing in MM, and supplemented with 2% (w/v) of glucose (○); 2% (w/v) of glycerol (■), 2% (w/v) of mannitol (□), and YEPD (●) at 30ºC, initial pH of 5.0 and at different salt concentrations. **B -** Stationary phase dry weight of cells described in A. **C**- Exponential variation of lag phase values as a function of medium salt concentration.

Osmotolerance has been reported to be also related, in *S. cerevisiae*, with the type of carbon source used and with the transition between respiratory and respiro-fermentative metabolism [Blomberg *et al*., 1988]. In fact, some of these aspects may apply to *C. halophila*, since their maximum tolerance level to NaCl has decreased in YEPD medium in comparison to MM with glucose as carbon source, being respectively 3.5 and 4.5 (Figure 2.8-A). For instance, YEPD is a complex medium, in which, at least, the nitrogen source and the micronutrients available are different from MM and thus the medium composition may be one of the aspects, affecting *C. halophila* growth in the presence of salt. On the other hand, growth with glycerol as carbon source led to an increase in maximal tolerance level up to 5M (Figure 2.8-A and B), evidencing the importance of this compound as carbon source in salt media, in a similar way as it was stated by Blomberg and collaborators (1988) for *S. cerevisiae*.

These results have their counterparts in the lag phase values measured in each case, which were significantly higher in YEPD with increasing salt concentrations, since this showed to be the most restrictive media in terms of maximum resistance (Table 2.5 and Figure 2.8-C).

Table 2.5 - Lag phase values (hours) at different salt concentrations of *C. halophila* cells growing in MM or YEP and supplemented either with 2% (w/v) of glucose; 2% (w/v) of glycerol, 2% (w/v) of mannitol, and YEPD at 30ºC with an initial pH of 5.0 .

[NaCl] (M)	Glucose-MM	Glucose-YEPD	Glycerol-MM	Mannitol-MM
0	2	2	2	2
0.5	2	4	2	2
1.0	2	4	2	2
1.5	4	7	2	2
2.0	4	10	2	4
2.5	7	15	4	10
3.0	20	50	14	24
3.5	30	168	25	96
4.0	150	n.g.	100	240
4.5	700	n.g.	370	n.d.
5.0	n.g.	n.g	n.d	n.d.

n.g. no growth; **n.d**. not determined

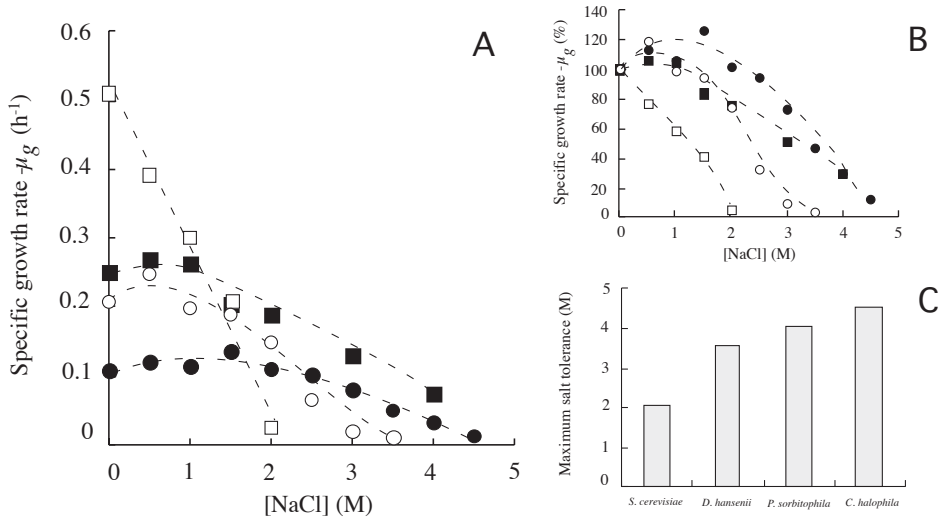
The slight increase observed in growth rate up to 2M NaCl was not accompanied by the respective values of dry weight in stationary phase, which instead showed a progressive decrease up to 2M of salt, followed by a small increase up to 4M (Figure 2.8-B). These results are though not surprising if we take in consideration that under stress, the cells will, most probably, deviate their main metabolism for the production and accumulation of osmolytes and for active extrusion of ions. This way, less energy will be available for growth, with an impact in the specific growth rate and biomass production. Since *C. halophila* is highly resistant to sodium chloride, the effect in growth rate is only visible for salt concentrations above 2.5M, although the impact of salinity in stationary dry weight is visible at any salt concentration (Figure 2.8-A and B). Curiously, the dry weight decreases in the range of salt concentrations where the growth rate is not negatively affected (*i.e.*, < 2.5M), and increases slightly when the growth rate is smaller (*i.e.*, > 2.5M) (Figure 2.8-B). Somehow, up to 2.5M

NaCl, metabolism is faster but partially deviated from biomass production, while for salt concentration above 2.5M, the growth was slower but was not accompanied by a reduction in biomass production. It is possible to speculate that cells may be metabolising very differently in these two situations, and this is probably related with the intrinsic salt tolerance mechanisms of each microorganism.

Temperature can also influence the physiological response under salt stress. Tokuoka (1993) assayed *Z. rouxii* in salt containing media at several temperatures and observed that the maximum temperature of growth increased with the reduction of the water activity of the medium. On contrary, Prista and Madeira-Lopes (1995) observed in *D. hansenii*, a reduction in the maximum temperature of growth with increasing salt media concentration. *C. halonitratophila* and *Z. nectarophilus* showed also to have a better performance in the presence of salt at 30ºC which was lost at 20ºC [Brown, 1976]. Though aware of the influence of temperature on salt stress tolerance behavior of each yeast, *C. halophila* was not assayed in this regard.

According to the survey made by Lages and collaborators (1999), *C. halophila* was the most salt-tolerant yeast from a larger group tested. This group was classified into four classes of tolerance according to four increasing molar steps of NaCl concentration in the medium. We compared growth of *C. halophila* with growth, under the same circumstances, of three other yeasts, each representing a different tolerance class: *P. sorbitophila* (4M), *D. hansenii* (3.5M), and a wild type strain of *S. cerevisiae* (2M) (Figure 2.9).

Figure 2.9. A- Specific growth rates (μ_g) of *C. halophila* (●), *D. hansenii* (○), *P. sorbitophila* (■) and *S. cerevisiae* (□), growing in MM supplemented with 2% (w/v) of glucose at 30ºC, in the presence of salt concentrations up to 4.5 M, without inoculum pre-adaptation. **B-** Comparison of specific growth rates expressed as percentage of the value obtained in the absence of salt. **C-** Maximum salt tolerance of each strain according to Lages *et al*., (1999), Neves *et al*., (1997) and the present work.



When there is no salt, *S. cerevisiae* grows faster than other more salt-tolerant yeasts, but it showed the steepest decrease in growth rate in the presence of increasing salt concentrations. On the opposite side, *C. halophila*, the slowest yeast in the absence of salt, was the less affected by its presence. When growth rate values were expressed as percentage of the value obtained in the absence of salt, *C. halophila*, appears clearly as the less affected yeast (Figure 2.9-B). Although highly salt resistant too, *D. hansenii* and *P. sorbitophila* have their growth rates affected more severely for lower concentrations of salt than *C. halophila* (Figure 2.9-A and B). This behavior seems to be, apparently, a general finding according to Anand and Brown (1968), in which the most osmotolerant yeasts had a maximal growth rate substantially lower than that of the non-tolerant strains. Nevertheless, no relevant explanation has been given so far for this interesting finding, although it is probable that this might be related with the halotolerant yeast metabolism that is highly directed to osmolyte synthesis and ions extrusion and thus deviated from biomass production and consequently from proliferation.

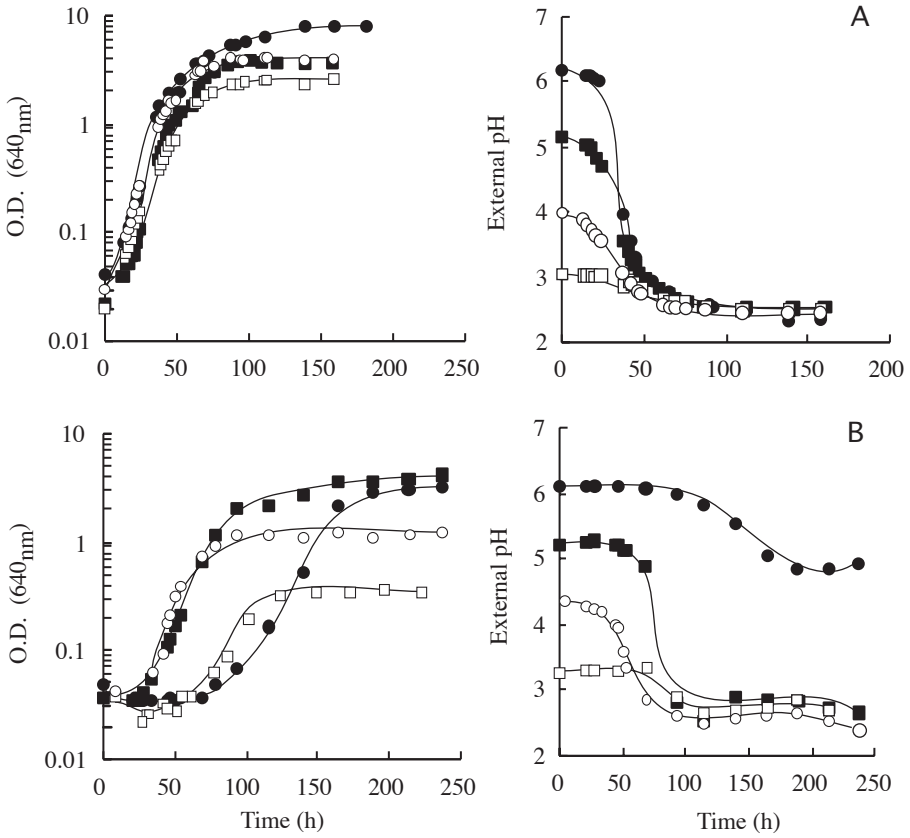
2.2.3.1. *Growth at different initial pH*

Yeasts have an ability to survive in a wide range of pH values, as mentioned above. Their growth and proliferation, namely in salt stress conditions, is though influenced by the external pH value. For instance, *C. versatilis* [Watanabe *et al*., 1993a], *Z. rouxii* [Watanabe *et al*., 1993b, 1995; Nishi and Yagi, 1995] and *S. pombe* [Jia *et al*., 1992] are examples of yeasts in which stress resistance revealed to be dependent on medium pH. Furthermore, the same authors reported that the plasma membrane H^+-ATPase, which is highly implicated in ion homeostasis in yeasts, is, in *C. versatilis* [Watanabe *et al*., 1993a] and *Z. rouxii* [Watanabe *et al*., 1991], stimulated *in vivo* by the presence of NaCl, but apparently independent of glucose metabolism. This behavior is very different from *S. cerevisiae* [Serrano, 1983] in which case it is stimulated by a glucose pulse and not by salt. At very high external salt concentrations, the proton motive force depends heavily on internal ion homeostasis and thus, on the transport systems the cell uses to control intracellular concentrations of sodium and/or potassium [Ramos, 1999]. Considering all this information we decided to search, in *C halophila* cells, the relation between initial medium pH and the salt concentration, and repeated the same type of growth assays as before but in media at four different initial medium pH, ranging from 3 to 6. Figure 2.10 presents as example the type of growth curves and the pH variation in cells of *C. halophila* growing without and with 3M of NaCl. Since the assays were made using batch culture systems, the initial pH of the medium suffered, as expected, significant changes during growth.

Usually, yeasts cells growing actively acidify the extracellular medium pH, essentially at the expense of the intense proton pumping made by the plasma membrane H^+-ATPase [Salhany *et al*., 1975; Serrano, 1978, 1983; Eraso and Gancedo, 1987]. It is obvious that, even if cells have good homeostatic systems, there will be an external medium pH at which cells will be no more capable to maintain internal pH. This happens frequently at pH values lower than 2.5 [Salhany *et al*., 1975]. At such external lower pH, the internal pH will drop and the enzymes will be inhibited and consequently the metabolism and growth is cessed. As can be observed in Figure 2.10, the final pH achieved at the end of exponentially growth phase was approximately 2.5 in all cultures, with the exception of the one starting at pH 6 in the presence of 3M of salt, in which the final pH did not get lower than 4.8. In fact, *C. halophila* cells entered stationary phase exactly when the extracellular medium pH lowered to 2.5 (Figure 2.10). The same type of phenomenon occurred for initial medium pH of 6 in the presence of 3M, but in this case the external pH at which cells enter stationary phase was 4.8. As will be seen further ahead, at initial

pH of 6 the carbon source is completely exhausted from the growth medium, and therefore it is unclear if the higher pH value obtained at the end of exponential phase is both due to the exhaustion of the carbon source and a higher medium buffer capacity in the presence of such NaCl concentrations or if it has an interpretation in a possible particular metabolism occurring at high salinities. This behavior at pH 6 was also observed in cells growing in the presence of 2.5M NaCl (not shown).

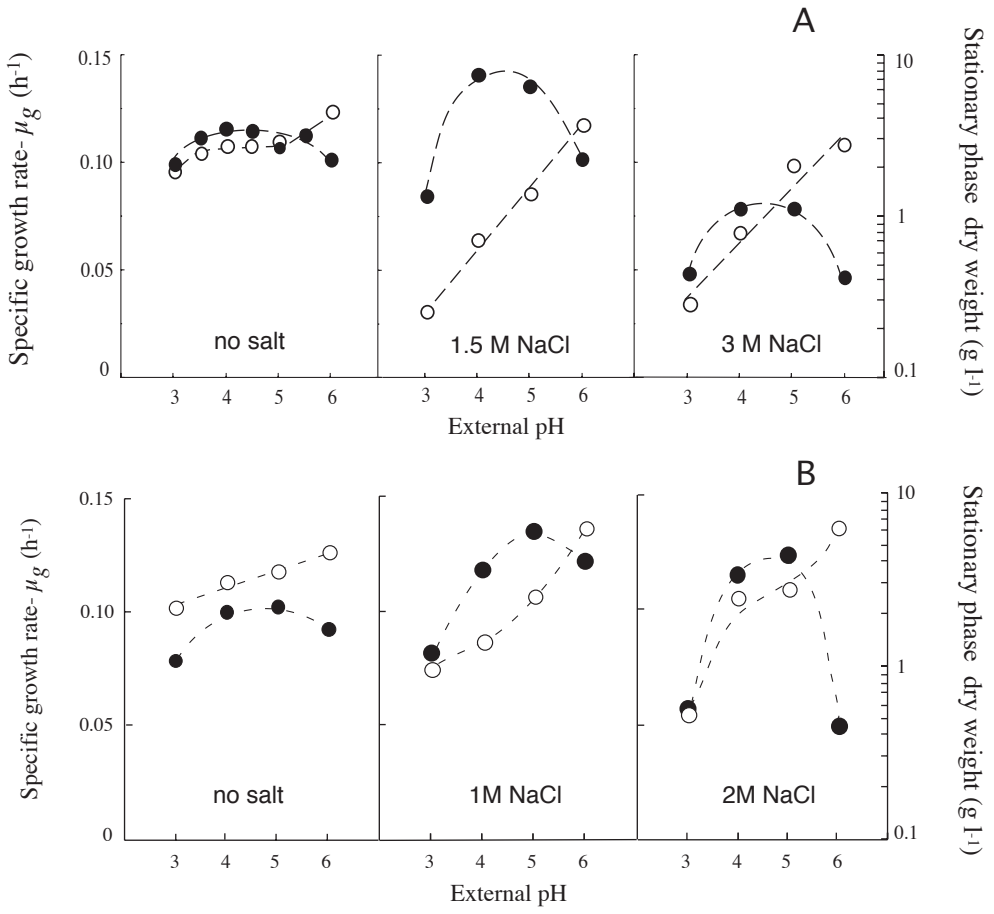
Figure 2.10. Growth curves and medium pH variation of *C. halophila* growing in MM supplemented with 2% (w/v) of glucose, at 30ºC, in the absence (**A**), and in the presence (**B**) of 3M of NaCl, at four initial medium pH values ranging from 3 to 6. Symbols: pH 3 (□), pH 4 (○), pH 5 (■), pH 6 (●).



For a better elucidation of the relation between growth/initial medium pH, glucose and glycerol results were organized in three different ways using two different carbon sources: (i) cells growing in the absence of salt, (ii) cells growing in the presence of 1.5M of NaCl (salt concentration at which *C. halophila* presented the highest growth rate), and (iii) cells growing in the presence of 2M and 3M NaCl (Figure 2.11). Similar results were found in cells growing in the absence and in the presence of salt using glucose or glycerol as carbon and energy source (Figure 2.11-A and B). Results

clearly show that the growth optimum pH ranges between 4 and 5, although in the absence of salt, growth rate values varied little in the pH range tested. This interval of pH is quite restrictive in the presence of salt even at salt concentrations that enhanced growth rate such as 1-1.5M. At pH values lower than 4 and higher than 5 the growth rate is highly affected (Figure 2.11-A and B). Most fungi are little affected by pH over a broad range, commonly 3 to 8, but usually yeasts prefer lower pH values, around 5, for optimum growth performances [Walker, 1998; Pitt and Hocking, 1997].

Figure 2.11. Comparison of specific growth rates (●) and stationary phase dry weight (○) of *C. halophila* cells grown in the absence, and in the presence of 1.5, 2 and 3M NaCl, at four initial medium pH values ranging from 3 to 6, in MM supplemented with **A**- glucose 2% (w/v), and **B**- glycerol 2% (w/v), at 30ºC.



Yet, pH/salt relations are still poorly investigated. To our knowledge, the first reports on the relations between pH and medium a_w were reported by English (1954), which showed that the sugar-tolerant *Z. rouxii* was able to grow in a wide pH range of 1.8-8.0 in the presence of high concentrations of

glucose. However, in the presence of 1M and 2 M NaCl, the pH range reduced to 3.0-6.0 and 4.0-5.0, respectively [Onishi, 1957; Yagi, 1988]. In fact, the reduction of the pH range allowing growth with a_w medium decrease is often encountered in yeast food spoilage behavior [Pitt and Hocking, 1997]. The same type of results were also obtained with *D. hansenii*, that, in spite of being able to grow at pH up to 8.5 in the absence of salt [Norkrans, 1966], a reduction in growth rate is observed in the presence of increasing salt concentrations in the media [Sørensen and Jakoben, 1997].

As can be observed in Figure 2.11-A and B, dry weight values increased exponentially with increasing medium pH values when using both carbon sources. At pH 6 the growth rate was smaller (more deeply in salt grown cells), but the dry weight value was the highest. Furthermore, the dry weight value at initial medium pH of 6 was almost unaltered in the absence as in the presence of salt and when using both carbon sources (Figure 2.11-A and B). According to these results, we performed assays in an extensive number of salt/initial medium pH combinations. Growth rates, stationary phase dry weights and total glucose consumption were determined in *C. halophila* cells grown at initial medium pH values ranging from 3 to 6 in media containing increasing salt concentrations from 0 to 4.5M (Figure 2.12.). Results were presented in two distinct manners for a better understanding, as a function of salt media concentration (Figure 2.12-A and B) and as a function of initial medium pH (Figure 2.12-C, D and E). As can be seen in Figure 2.12-A and C specific growth rates vary with the medium pH and with the salt concentration as showed before Figure 2.8-A and in Figure 2.11. However, Figure 2.12 complements the Figure 2.11 by showing the effect of pH in growth rate pattern over a wider range of salt concentrations. The pattern of μg variation with salt is identical for all culture initial pH assayed (Figure 2.12-A). On the other hand, when the same results are plotted as a function of initial pH, instead of salt concentrations (Figure 2.12-C) it becomes obvious that the pH range allowing growth in *C. halophila* cells is reduced for high salt concentrations, being of 4.0-5.0 at 4M NaCl instead of 3.0-6.0. Variations in dry weight obtained in stationary phase at several pH and salt concentrations are more pronounced than μg´s (Figure 2.12-B and D). As discussed before for Figure 2.11, dry weight results at pH 6 present the highest values even at the maximum salt concentration allowing growth at this pH (3.5M NaCl), but the respective growth rates are quite reduced. Even at pH 5 in which growth rates have their best values, dry weight was lower than at pH 6. Nevertheless, for high salt concentrations dry weight values tend to approximate (Figure 2.12 B). As discussed before for Figure 2.10, this result might be related with the reduction of the medium pH throughout the cell growth, which at pH 6 does not drop beyond 4.8. Although there is no literature supporting this hypothesis, we may speculate that media having both high pH values and high salt concentration have, most probably, a different buffering capacity and this phenomenon allows cells to extend their growth. Low medium pH is not achieved and consequently growth is not impaired. Indeed, this hypothesis may have some support in the results presented in Figure 2.12-D, which shows the glucose consumption at different pH/salt concentrations. As can be seen, only cells growing at pH 5 and 6 and at salt concentrations above 2.5 M NaCl, showed a complete consumption of the glucose available for growth. Whether cells grown in the presence of high salt concentrations are requiring extra carbon source consumption and thus use completely the available carbon source, or if the initial medium pH value is simply allowing the exhaustion of the carbon source, can not be discriminated in batch systems with varying medium pH, but rather in continuous culture studies were not performed.

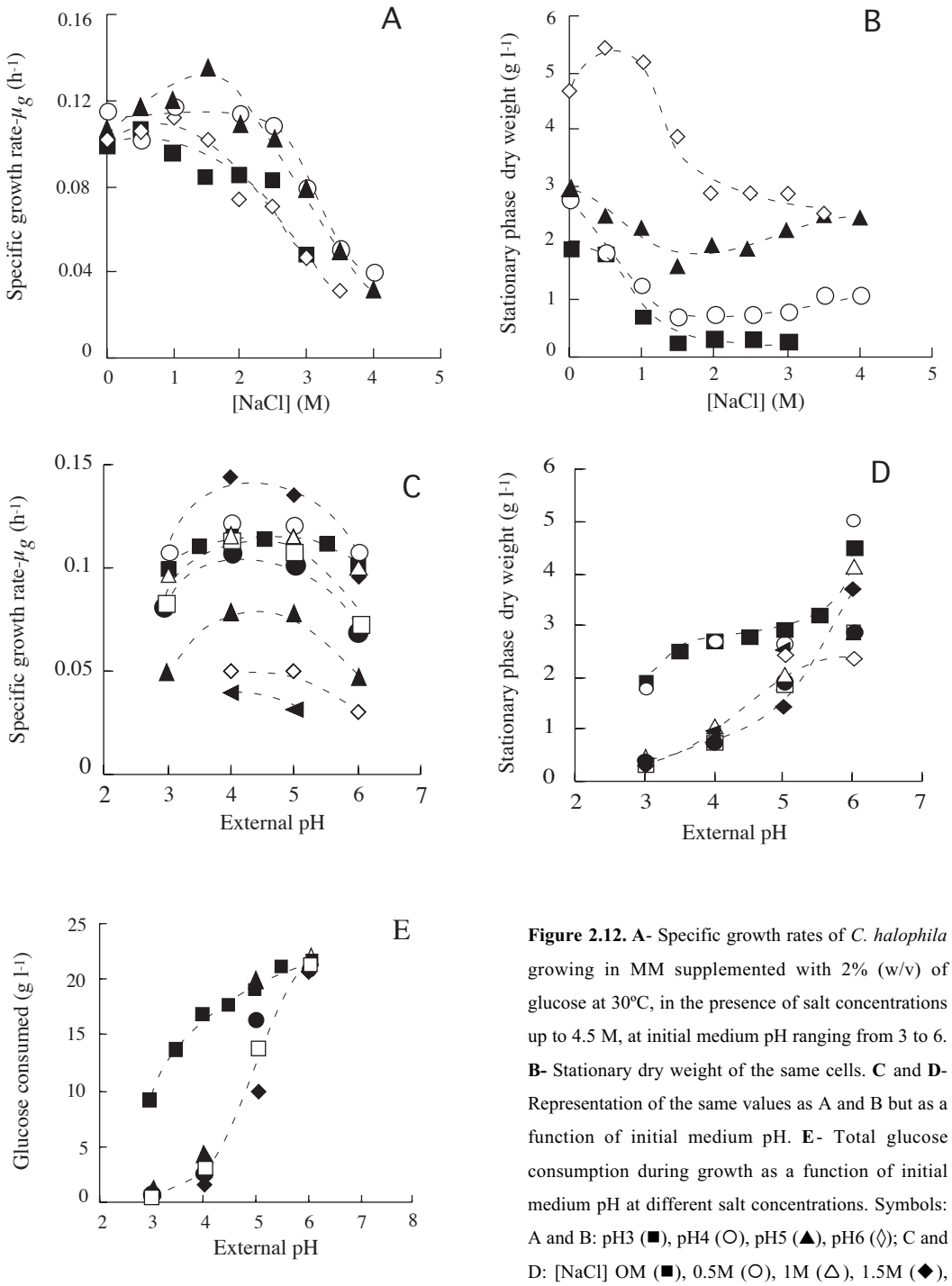


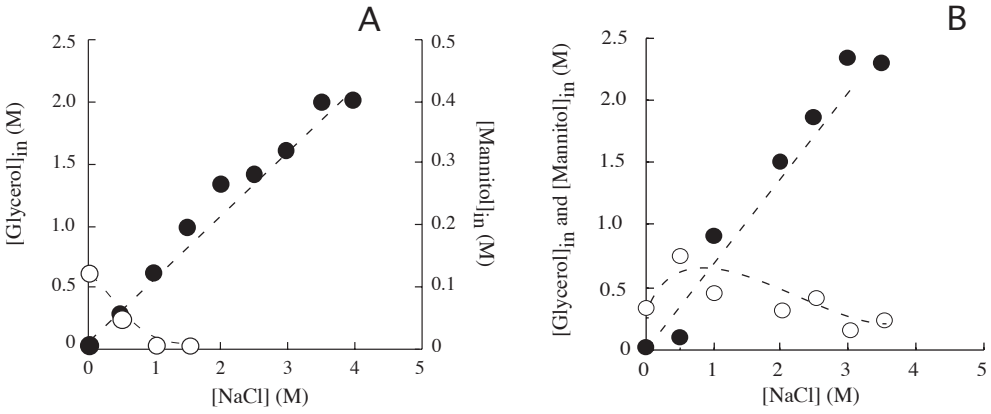
Figure 2.12. A- Specific growth rates of *C. halophila* growing in MM supplemented with 2% (w/v) of glucose at 30ºC, in the presence of salt concentrations up to 4.5 M, at initial medium pH ranging from 3 to 6. **B-** Stationary dry weight of the same cells. **C** and **D**- Representation of the same values as A and B but as a function of initial medium pH. **E**- Total glucose consumption during growth as a function of initial medium pH at different salt concentrations. Symbols: A and B: pH3 (■), pH4 (○), pH5 (▲), pH6 (◊); C and D: [NaCl] OM (■), 0.5M (○), 1M (△), 1.5M (◆), 2M (□), 2.5M (●), 3M (▲), 3.5M (◊), 4M (◄).

2.2.4. *Intracellular solutes*

Compounds accumulated in C. halophila cells when growing in the presence of salt

Since *C. halophila* showed to be highly resistant to NaCl and accumulated naturally glycerol and mannitol, compounds with recognized function as compatible solutes in other yeasts, we searched the type of compounds accumulated in the presence of increasing salt concentrations. Cells of *C. halophila* growing actively in the presence of different salt concentrations were collected in the late exponential phase and analysed for their intracellular composition (Figure 2.13).

Figure 2.13. Intracellular polyol accumulation of *C. halophila* cells grown at late exponential phase in MM at 30ºC supplemented with increasing salt concentrations using **A**- glucose 2% (w/v), and **B**- mannitol 2% (w/v) as carbon and energy sources. Symbols: Glycerol (●) and Mannitol (○).



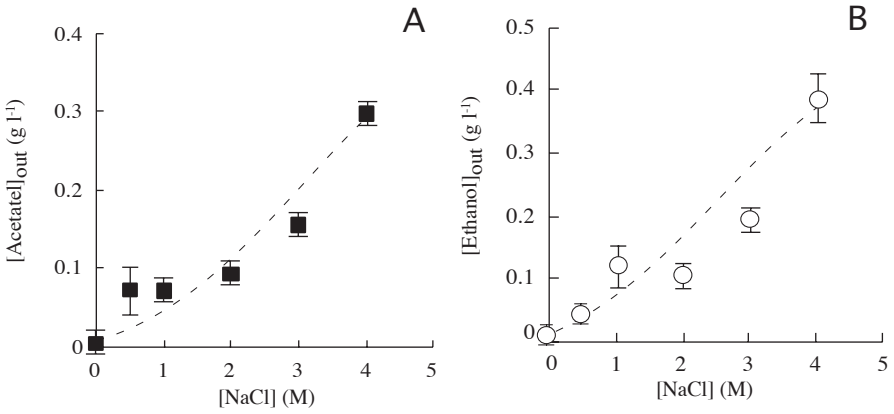
Cells of *C. halophila* growing on glucose in the absence of salt stress accumulate approximately the same amounts of glycerol and mannitol (≈ 0.2 µmol mg $^{-1}$ d.w.). When growth was performed in the presence of increasing salt concentrations, up to a maximum of 4M the intracellular amounts of glycerol increased in a linear relation with the medium salinity (Figure 2.13-A). On the other hand, mannitol was detected intracellularly in lower values, and decreased progressively with increasing salt concentrations, being undetected for salt concentrations above 1.5 M NaCl. No other compounds were detected with the methodological approaches used. Clearly glycerol is functioning under salt stress as compatible solute. Mannitol instead appears to be unrelated to salt stress response. This became more obvious when results in glucose growing cells (Figure 2.13-A) were compared with those of mannitol growing cells (Figure 2.13-B). Salt-mannitol grown cells accumulated glycerol intracellularly at identical levels as salt-glucose grown cells (Figure 2.13-A and B). It should also be noted that mannitol is not only accumulated intracellularly in salt-mannitol grown cells at higher values than in salt-glucose grown cells but is also detected in cells growing at higher salt concentrations, being

still detected at 3.5 M NaCl (Figure 2.13-A and B). Nevertheless, intracellular concentration of mannitol decreases with increasing salt concentrations regardless of the carbon source used. It is likely that the intracellular concentrations of mannitol detected in salt-mannitol grown cells are due to the accumulation of this compound inside the cell as a consequence of its uptake system, together with inefficiency or delay in this substrate consumption. As can be seen in Table 2.4 mannitol was detected in several other yeasts but no specific function has yet been attributed to this compound, except for some filamentous fungi that accumulate mannitol for osmoregulatory purposes. Thus, mannitol remains without a clear function in yeasts metabolism.

The amounts of extracellularly accumulated compounds on cultures of *C. halophila* growing on salt were monitored (not shown). Glycerol was not found, indicating an efficient retention for osmoregulation purposes. Mannitol was progressively released into the medium according to growth progression, consistently with the decrease of this compound measured intracellularly.

C. halophila cells growing in glucose as carbon source produced naturally acetate and ethanol (Figure 2.4), although acetate was only detected intracellularly transiently and at low concentrations while ethanol was excreted to the medium at high concentrations. When in the presence of increasing salt concentrations (Figure 2.14), *C. halophila* cells produced and excreted to the medium acetate and ethanol.

Figure 2.14. Extracellular accumulation of **A**- acetate and **B-** ethanol, in *C. halophila* cells grown at late exponential phase in MM at 30ºC supplemented with increasing salt concentrations with 2% (w/v) of glucose.



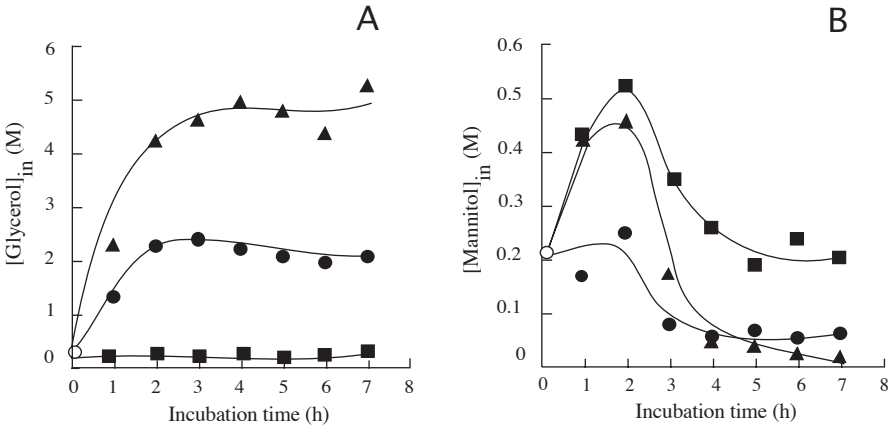
As can be seen in Figure 2.14 ethanol and acetate concentrations increased almost linearly with the salt concentration in the medium. These compounds were only detected extracellularly, which indicates that they are immediately lost by the cell to the medium. The reason why acetate and ethanol increased with salt in the medium can only be suggested. While ethanol production is accompanied by NAD^+ production, acetate production leads to NAD(P)H generation, thus, usually

ethanol and acetate result from the competition between acetaldehyde dehydrogenase and alcohol dehydrogenase for acetaldehyde (Figure 2.1). Production of both compounds indicates that these may be fulfilling, in metabolic terms, different purposes. For example ethanol may be produced for neutral redox balance of glycoslysis and acetate for providing reduced co-factors. It was also reported that an attempt to increase glycerol production in *S. cerevisiae* led to an increase in acetic acid production [Remize *et al*., 1999], and thus the presence of acetate might be related with glycerol production under stress. Indeed, Zhuge and collaborators (2001) also reported acetic acid accumulation in *Candida glycerinogenes*, a strain producing high glycerol concentration in an aerobic process for glycerol fermentation. Since under salt stress conditions glycerol is used as compatible solute, its normal function in redox balance may be compromised and thus acetate may be instead produced to fulfill this function.

Compounds accumulated in C. halophila cells when salt-shocked

Cells of *C. halophila* were also submitted to salt shock using three salt concentrations, 1, 2 and 3 M NaCl. Intracellular compounds accumulation was followed during seven hours of incubation time (Figure 2.15). It was possible to detect intracellularly five distinct compounds: glycerol, mannitol, acetic acid, ethanol and trehalose.

Figure 2.15. Intracellular accumulation of **A**- glycerol, **B-** mannitol, in *C. halophila* cells growing in MM supplemented with 2% (w/v) of glucose, at 30ºC and shocked for a period of seven hours with three different salt concentrations: 1M NaCl (●), 2M NaCl (▲) and 3M NaCl (■)

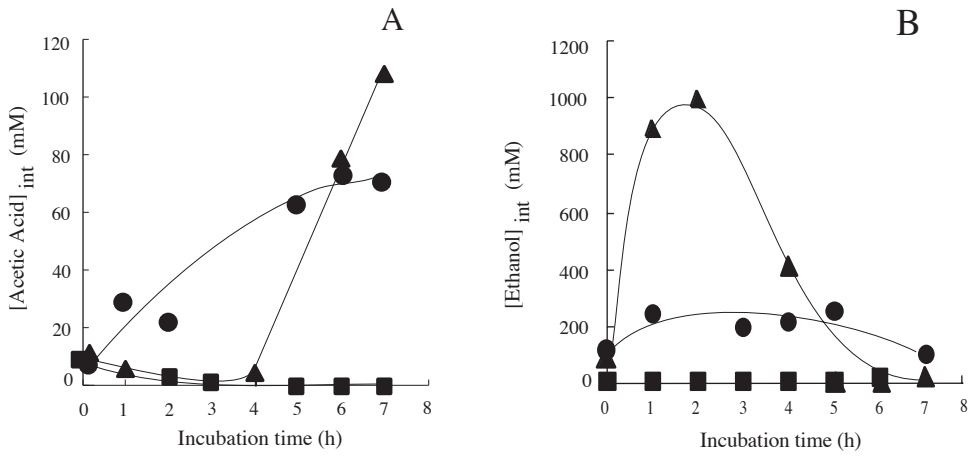


In cells shocked with 1M or 2 M NaCl glycerol accumulation stabilized after about 2 hours, while, for cells incubated in 3 M NaCl, after 7 hours it still had not begun (Figure 2.15-A). It should be noted that the intracellular glycerol values obtained after 7 hours in cells shocked with 1M and 2M NaCl (Figure

2.15-A) are several times higher than the values found for the same salt concentrations in cells growing exponentially in the presence of salt (Figure 2.13-A). This comparison led to us to postulate that internal composition is modulated during growth until a perfect internal homeostasis is achieved. In fact, the periods of time required for glycerol accumulation at 1 and 2 M NaCl did not correspond to the duration of the lag phase of growth curves under the same circumstances, which were, respectively 4 and 10 hours. *C. halophila* cells growing in the presence of 3M NaCl exhibited a lag phase of 20 hours. Since the shock assay lasted for 7 hours only, we do not know if the time required to glycerol accumulation is lower than the lag phase value at this salt concentration as observed for 1 and 2 M NaCl. Equivalent results have been reported before for *S. cerevisae* [Albertyn *et al*., 1994a,b]. These authors showed that *S. cerevisiae* cells submitted to a salt shock of 1.2 M NaCl exhibited a threefold increase in glycerol content after 1 hour incubation, which did not correspond to lag phase duration. This was accompanied by an increase in the specific activity of glycerol-3-phosphate dehydrogenase (Gpdp), the key enzyme in glycerol production. According to the same authors GPDH activity started to increase immediately after the salt shock, being ten times higher after six hours incubation. Furthermore a linear relation between Gpdp activity and salt concentration was also reported [Albertyn *et al*., 1994a,b].

Increased glycerol accumulation was followed by an opposite reduction in the mannitol content (Figure 2.15-B). Mannitol disappearance was faster for cells accumulating more glycerol and much slower for the cells with delayed glycerol production (Figure 2.15-B). The variation of intracellular amounts of mannitol was consistent with the results obtained in cells growing exponentially in salt medium (Figure 2.13-A). Besides, mannitol concentration was higher in cells exposed to 2 and 3M NaCl. The reason underlying this temporary increase is not clear. Whatever the function this compound is having in *C. halophila* cells, it does not seem to be related with salt media. Acetic acid and ethanol were accumulated only in cells shocked with 1 and 2 M NaCl (Figure 2.16).

Figure 2.16. Intracellular accumulation of **A-** acetic acid and **B** ethanol, in *C. halophila* cells growing in MM supplemented with 2% (w/v) of glucose, at 30ºC and shocked for a period of seven hours with three different salt concentrations: 1M NaCl (●), 2M NaCl (▲) and 3M NaCl (■)



This is another indirect indication that *C. halophila* in the presence of 3M NaCl takes more than 7 hours to adjust to stress and reactivate metabolism. A closer look of Figure 2.16 presenting results on ethanol and acetate accumulation highlights the opposite fitting of these two compounds. In fact, when ethanol concentration decreased, acetate concentration increased. This result could be easily explained considering that while ethanol is being produced, no acetaldehyde should be diverted to acetate production, but when ethanol production is diminished, then acetate production should increase, assuming that carbon flux through glycolysis maintains. Nevertheless the regulation of this metabolic branch point is not that simple, in particular in cells either shocked or grown on salt. Although ethanol increase is apparently transient in shocked cells (Figure 2.16), in fact it keeps being produced and excreted to the medium in increasing amounts for increasing salt concentrations when cells are grown on the same salt concentrations (Figure 2.14). In addition, both internal and external acetate increase under salt stress (Figure 2.14 and Figure 2.16).

Trehalose was found to accumulate to approximately identical values at all salt concentrations tested and at all incubation periods considered (Figure 2.17).

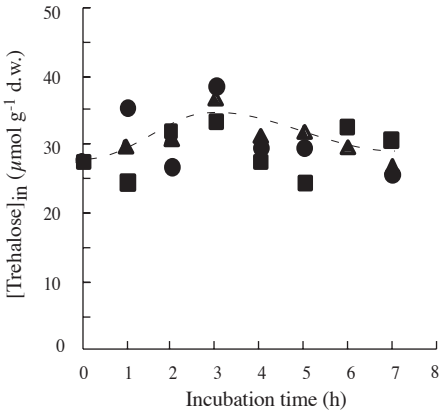


Figure 2.17. Intracellular accumulation of trehalose, in *C. halophila* cells growing in MM supplemented with 2% (w/v) of glucose, at 30ºC and shocked for a period of seven hours with three different salt concentrations: 1M NaCl (●), 2M NaCl (▲) and 3M NaCl (■)

Intracellular values need to be carefully presented, since if we take into account the intracellular volume of *C. halophila* salt shocked cells, in the case of trehalose, it will give the impression that cells have higher amounts of trehalose in salt shocked cells, which is merely the effect of cell volume reduction. Thus, trehalose values were not indexed to intracellular values as the other compounds shown before. This artefact is not visible with the other compounds detected intracelullarly because they effectively varied their intracellular concentrations in the presence of salt. Thus, it is obvious that trehalose does not accumulate according to stress response. This is compatible with a role as reserve carbohydrate, stabilizer or is a result of an eventual futile cycle. Ölz and collaborators (1993) reported in *S. cerevisiae* trehalose intracellular content in *S. cerevisiae* growing in chemostats at different dilution rates in the presence and in the absence of salt. A similar pattern was obtained at all conditions, trehalose content decreasing with increasing dilution rates. This was more pronounced for higher salinities. For this reason trehalose in *S. cerevisiae* has not been

associated with osmotic response. Nevertheless *S. cerevisiae*, when shocked with 1.4M NaCl takes approximately 7 hours to initiate high intracellular concentrations of glycerol, while in the same period intracellular trehalose increased transiently, since in the beginning of glycerol accumulation, values were again, lower than the control [Singh and Norton, 1991].

2.2.5. *O_2 consumption and CO_2 production rates*

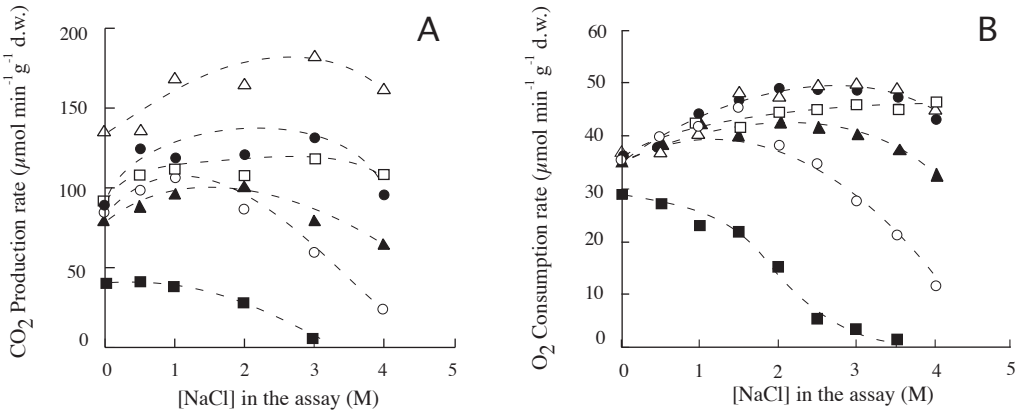
Biochemical pathways in yeasts may be regulated at various levels. These include: enzyme synthesis (*e.g.*, induction, repression and derepression of gene expression), enzyme activity (*e.g.*, allosteric activation, inhibition or interconversion of enzymes) and cellular compartmentalization (*e.g.* mitochondrial localization of respiratory enzymes). Yeasts exhibit diversity in their modes of energy generation and Table 2.1 presented in this Chapter introduction categorized some groups of yeasts with respect to their utilization of respiration and fermentation in ATP production. Of the environmental factors that regulate respiration and fermentation in yeast cells, the availability of glucose and oxygen are the best documented. These factors are linked to the expression of several regulatory phenomena such as Pasteur, Crabtree, Custers and Kluyver effects, as mentioned before. Note, however, that yeasts adapt to varying environments, so the manifestation of a particular effect will also depend on the prevailing growth conditions. For instance, *S. cerevisiae* may evidence either a respirative, respirofermentative or fermentative metabolism depending on the type of culture system used, and the oxygen, carbon and nitrogen availability in the medium. This is in agreement with what was published by Fiechter and Seghezzi (1992), which recommended the use of the effects terminology referred above with care, since they define a single state of certain cultures under specialized conditions and not a general metabolic phenomenon.

C. halophila cells cultivated at 30ºC in MM supplemented with 2% (w/v) glucose and with an 2:1 air:volume ratio respired and fermented simultaneously (Figure 2.18). O_2 consumption rate was 29.4 μmol min^{-1} $d.w.^{-1}$ and CO_2 production rate was 37.8 μmol min^{-1} $d.w.^{-1}$. Under these experimental conditions *C. halophila* could thus be considered a respirofermentative, Crabtree positive yeast. In the presence of increasing salt concentrations, and maintaining culture conditions, it kept behaving as a respirofermentative yeast (Figure 2.15). This was true for all combinations of salt concentrations from 0 to 4M in growth medium and in assay conditions tested (Figure 2.18). These results are quite complex since we have to consider that every culture grown at a certain salt concentration when assayed at higher one is actually suffering an osmotic up-shock, while if it is assayed at a lower salt concentration it is suffering an osmotic down-shock.

The pattern of variation is though quite constant, exception made of the cells cultured without salt, which do decrease more steeply both fermentation and respiration rates for salt concentrations rather moderate for *C. halophila* standards (Figure 2.18). Nevertheless, when the results obtained with the same salt concentration in growth medium and assay where compared (Figure 2.19), a pattern arose, indicating a clear increase in CO_2 production rates in comparison with the almost maintenance of O_2 consumption rates. If we consider the results obtained before concerning ethanol production under salt stress (Figure 2.14), it is clear that the CO_2 production rates obtained are the result of enhanced fermentation. Ethanol concentration, as expected, increased progressively with salt in the medium and he amount of extracellular ethanol measured in cells cultivated in 4M NaCl was ≈400 % of the values observed in cells cultivated without salt (Figure 2.17). This percentage fitted the increase observed in

CO_2 production rates (Figure 2.19). Together, the results presented confirm that *C. halophila* behaves as respirofermentative yeast and that fermentation has an important role under salt stress conditions.

Figure 2.18. Carbon dioxide production rates (**A**) and Oxygen consumption rates (**B**) of *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose at 30ºC, in the absence and in the presence of increasing salt concentrations, and assayed at varying salt concentrations. **Symbols**: NaCl in the medium 0M (■), 0.5M (○), 1M (▲), 2M (□), 3M (●) and 4M (△).



Ölz and collaborators (1993) published some results concerning the growth of *S. cerevisiae* in chemostat in the presence of salt. They verified that salt stress cells have to initiate their fermentative metabolism at a lower dilution rate, in which metabolism was entirely respiratory in the absence of salt, in order to meet the demand for an increased rate of energy production during growth at high salinity. Usually this leads to a drastic yield reduction.

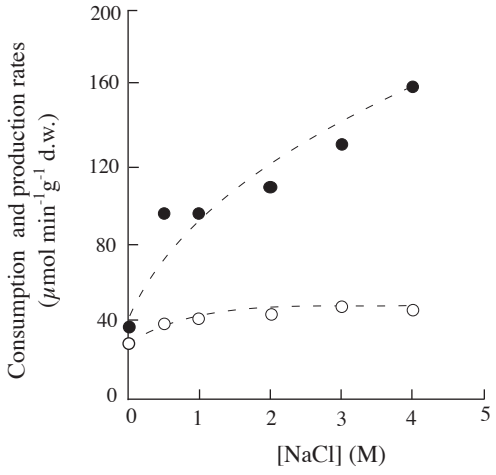


Figure 2.19. Carbon dioxide production rates (●) and oxygen consumption rates (○) of *C. halophila* cells grown in MM supplemented with 2% (w/v) glucose at 30ºC, in the absence and in the presence of increasing salt concentrations, and assayed at the same salt concentration as in the medium growth.

C. halophila results concerning CO_2 production and O_2 consumption rates are quite unique. There are few published results of this kind of nature. Norkrans (1968) reported studies of respiration and fermentation rates in marine occurring yeasts such as: *D. hansenii*, *S. cerevisiae*, *Cryptococcus albidus* and *Candida zeylanoides*. All the yeasts assayed showed reduced fermentation and respiration rates at increasing salt concentrations in the medium. *D. hansenii* showed to be most resistant to NaCl and although markedly reduced, respiration and fermentation rates were still measurable at 4M NaCl. This result was later confirmed by Neves and collaborators (1997) who also demonstrated that *D. hansenii* indeed presents reduced fermentation and respiration rates with increasing salt concentrations. Such high respiration and fermentation values at salt concentrations like 4M are consistent with the extreme halotolerant character of *C. halophila*, since they indicate that metabolism is optimized in order to overcome such aggressive environments.

2.3. Concluding remarks

To conclude, we recapitulate the main physiological results obtained in the yeast *C. halophila* in this chapter:

- Ability to grow up to 4.5M NaCl in MM with glucose as carbon source, or 5M with glycerol as carbon source
- Improvement in growth rate up to 2M NaCl, independently of the carbon source used.
- Sequential utilization of glucose and glycerol, and simultaneous utilization of glycerol and mannitol in mixed media.
- Production and accumulation of glycerol, mannitol and trehalose in the absence of salt
- Accumulation of high intracellular glycerol concentrations in cells growing in the presence of salt, according to a compatible solute role
- Mannitol not accumulating in response to salt stress - function remaining unknown.
- Respirofermentative (Crabtree positive) metabolism
- Enhanced fermentation and respiration rates under salt stress