Ann Microbiol (2012) 62:1345–1351
DOI 10.1007/s13213-011-0381-8

ORIGINAL ARTICLE

Impact of an osmotic stress on the intracellular volume of *Hansenula anomala*

Hayet Djelal · François Larher · Guy Martin · Abdeltif Amrane

Received: 17 August 2011 / Accepted: 28 October 2011 / Published online: 15 November 2011

Abstract The effect of an osmotic stress resulting from high glucose or NaCl concentrations on the morphology and intracellular volume of *Hansenula anomala* was examined by scanning electronic microscopy and changes in the intracellular volume accessible to tritiated water, respectively. No noticeable change in the cell morphology was observed, with the cells remaining ellipsoidal. An increase in the contribution of compatible solutes, such as arabitol, glycerol and trehalose, to the cell volume, was not sufficient to counterbalance the decrease in the volume accessible to tritiated water for increasing water stress, leading to a decrease in cell volume. For a given morphology, a decrease in the cell volume allowed the cell to maximize the surface to ratio volume for a better distribution of the external osmotic pressure. It also allowed maximization of the compatible solute concentration (for a given amount of accumulated compounds), leading to an increase in the internal osmotic potential to counterbalance the osmotic potential of the surrounding medium. However, the accumulation of compatible solutes in the cell did not allow any adjustment of the osmotic potential of cells in high-osmolarity medium, especially in the case of NaCl as the osmoticum, thereby confirming the higher stress effect of salt relative to glucose.

Keywords Compatible solutes · *Hansenula anomala* · Intracellular volume · Osmotic stress

H. Djelal · G. Martin · A. Amrane (✉)
Ecole Nationale Supérieure de Chimie de Rennes,
CNRS, UMR 6226,
Avenue du Général Leclerc, CS 50837,
35708 Rennes Cedex 7, France
e-mail: abdeltif.amrane@univ-rennes1.fr

H. Djelal · G. Martin · A. Amrane
Université européenne de Bretagne,
Rennes, France

F. Larher
UMR CNRS 6026 ICM Equipe OMS, Université de Rennes 1,
Campus de Beaulieu, Bât. 14, CS 74205,
35042 Rennes cedex, France

Introduction

Osmoregulation can be defined as the potential to maintain cell volume and turgor pressure following a change in the external water potential (Brown and Edgley 1980; Hohmann et al. 2007). The osmoadaptation of xerotolerant yeast growing in media of low water activity is characterized by a passive water outflow through the cytoplasmic membrane to restore thermodynamic equilibrium (Blomberg 2000) by Na^+ and K^+ inflow and outflow, respectively (Larsson et al. 1990) and by the synthesis and partial accumulation of small organic solutes, such as polyols, in the yeast cytoplasm (Adler and Gustafsson 1980; Brown and Edgley 1980).

Cell volume is a strain-specific function, but it also depends on the water activity of the culture medium (Rose 1975). Gustafsson and Norkrans (1976) reported that during exponential growth, the cell volume of *Debaryomyces hansenii* was found to decrease with increasing salinity of the culture medium. However, it has been reported that the intracellular volume of *Saccharomyces cerevisiae* does not vary linearly with medium osmolarity (Morris et al. 1986). No change in the cell-wall structure or the cytoplasmic membrane was suggested by these authors.

The intracellular volume of *Hansenula anomala* cells was determined by Van Eck (1988) by microscopic observation and by the diffusion of various labelled elements, and obtained a higher mean value using the

former method. Indeed, total cell volume can be deduced from microscopic observations, while the diffusion of tritiated water and [^{14}C]inulin gives the volume accessible to tritiated water in the cytoplasm. The use of [^{14}C]inulin and tritiated water to determine the intracellular (cytoplasmic) volume results in determination of the cell volume accessible to the lowest labelled molecule—and not the total intracellular volume. However, owing to the rapid balance between water protons and cellular components, such as glycerol and arabitol, the corresponding error is generally negligible, except in the case of a high accumulation of intracellular polyols (Katz and Avron 1985). To better estimate the intracellular volume, some authors add the volume occupied by glycerol and arabitol to the volume accessible to tritiated water (Larsson et al. 1990). It should also be noted that tritiated water cannot penetrate certain cellular components, such as fat globules, whose volume in the cytoplasm cannot be neglected (Van Eck 1988). Otherwise, organelles and storage granules constitute the non-osmotic volume (Meikle et al. 1988).

Reed et al. (1987) calculated the non-osmotic volume for different yeasts using the Boyle–Van't Hoff relation and found that it increased with increasing medium salinity. Under some culture conditions, the non-osmotic volume can constitute 28–53% of the total volume of *D. hansenii* cells (Reed et al. 1987). Increasing amounts of NaCl lead to an increased production of polyols, resulting in an increase in the non-aqueous part of cells (Larsson et al. 1990).

The aim of this study was to examine the effect of medium osmolarity on cell volume. The effect of high glucose or NaCl concentrations on the morphology and intracellular volume of *H. anomala* was examined by scanning electronic microscopy (SEM) and determination of the cytoplasmic volume accessible to tritiated water.

Materials and methods

Microorganism

The osmotolerant yeast *Hansenula anomala* var. *anomala* (CBS 5759; Centraalbureau voor Schimmelcultures, Delft, the Netherlands) was used in this study. *H. anomala* is also designated as *Pichia anomala*, *Wickerhamomyces anomalus* (Passoth et al. 2011), as well as *Candida pelliculosa* (Passoth et al. 2006). Stock cultures were maintained on a solid medium (Djelal et al. 2005).

Culture media

The synthetic medium, which contained 10 mmol L^{-1} NH_4Cl, 3.7 mmol L^{-1} KH_2PO_4, 4 mmol L^{-1} $MgSO_4{\cdot}7H_2O$ and an EDTA mineral solution derived from the Wickerham medium (Wickerham 1951), was supplemented with glucose and NaCl at the specified concentrations (Djelal et al. 2005). KOH 1 mol L^{-1} was used to adjust the pH to 5.5.

The range of glucose and NaCl concentrations tested were 0–2.2 mol L^{-1} (no NaCl added) and 0–2 mol L^{-1} (0.56 mol L^{-1} glucose), respectively.

Culture conditions

Duplicate cultures were inoculated as previously described (Djelal et al. 2006) and cultured in 500-mL Erlenmeyer flasks, at an agitation rate of 150 rpm. The working volume was 200 mL.

Irrespective of the mode of culture, 2 mL seed culture per litre of culture medium was used as inoculum, and the temperature was maintained at 28°C. Cells were harvested in the beginning of the stationary state.

Analyses

The identification and determination of metabolites were carried out on a high-performance liquid chromatography (HPLC) system equipped with an ion exclusion column (model HPX-87H, 300 × 7.8 mm; Bio-Rad, Hercules, CA) and maintained at 45°C (Oven Croco-Cil; Cluzeau–Info–Labo, Ste Foy La Grande, France). The flow rate was 0.6 mL min^{-1} (Waters, Milford, MA) using sulphuric acid 0.01 N as the mobile phase. A refractometer (model 7512; ERC, Saitama, Japan) was used for the detection of the different compounds. The standard solution contained glucose, glycerol, arabitol and trehalose, each at 5 mmol L^{-1}.

Final biomass concentrations were deduced from dry cell weight measurements.

Extraction of intracellular solutes

A 6-mL sample was centrifuged (4°C, 2200 *g*, 5 min). Cells were collected by centrifugation, followed by two washes in KCl (isotonic to the culture medium), before being suspended in 2 ml of ultrapure water. The obtained solution was heated at 100°C for 10 min, and the intracellular components released during cell autolysis were then recovered in the supernatant following centrifugation (4°C, 2200 *g*, 5 min) and used for intracellular glycerol analysis.

Measurement of the intracellular volume

Cells were incubated in the presence of two labelled molecules—a small molecule, namely, tritiated water, and a large one, inulin ([^{14}C]hydroxymethyl). Tritiated water (^3H_2O) can penetrate into the cell volume, whereas only inulin can only access the cell-wall and cytoplasmic spaces. The protocol used has been reported by Stock et al. (1977).

The cells were centrifuged (4°C, 1800 *g* for 5 min) and then resuspended in the same culture medium, but concentrated by tenfold. Then, 100 µL of inulin ^{14}C (128 kBq mL^{-1}; CEA, Saclay, France) and 100 µL of $^{3}H_2O$ (1260 kBq mL^{-1}; CEA) were added to 500 µL of the concentrated solution. Following mixing on a vortex mixer, this solution was incubated at ambient temperature for 30 min, then centrifuged (4°C, 1800 *g* for 5 min). A 100-µL sample of the supernatant was placed in a combustion chamber to be burnt for 30 s (oven oxidizer; model 306, Packard Instruments, Downers Grove, IL, USA). Of the remaining supernatant, 100 µL was added to the sedimented cells and, following mixing on a vortex mixer, 100 µL of the resulting mixture was burnt under the same conditions. The combustion products ($^{14}CO_2$ and $^{3}H_2O$) were separated in the oven oxidizer and the radioactivity measured by scintillation spectrometry (model 2200 CA; Packard Instruments, Downers Grove, IL). Two values were obtained for the supernatant and for the sedimented cells, resulting from [^{14}C]inulin and tritiated water ($^{3}H_2O$), respectively. The ratio of the two values recorded for each isotopic element gave the volume occupied by each radioactive compound, namely V_I and V_e respectively. The space inaccessible to inulin was $V=V_e - V_I$. The values given here are the average of ten measurements.

Scanning electron microscopy

Cells were fixed in 2.5 % glutaraldehyde for 5 h. The solution was then centrifuged (4°C, 1800 *g*, 5 min), and the sedimented cells were rinsed three times with 0.2 M phosphate buffer. For dehydration, the sedimented cells were immersed in an ascending series of acetone baths (70, 90 and 100%; 10 min each). Critical point drying was performed prior to SEM examination by progressively removing the acetone and replacing it with liquid CO_2; the subsequent decrease to atmospheric pressure allowed CO_2 evaporation. Samples were then covered with a thin gold film. The cells were observed using a JEOL JSM 35 microscope (JEOL SAS, Croissy-sur-Seine, France).

Cell volume measurement

Cell volume was determined using the following formulas (Van Eck 1988):

$$\text{For ellipsoidal cells}: \quad V = \frac{\pi}{6} ab^2 \tag{1}$$

where V is the total cell volume, a is the length of the small axis and b is the length of the long axis, and

$$\text{And for cylindrical cells}: \quad V = \pi \frac{d^2}{4} l \tag{2}$$

where D is the mean diameter and l is the cell length.

The values given here are the average of ten measurements.

Determination of the compatible solute concentrations

The intracellular compatible solute concentration C (mol L^{-1}) corresponds to the ratio of the intracellular compatible solute accumulation A [mmol g^{-1} dry weight (DW)] to the intracellular volume accessible to tritiated water V_e (mL g^{-1} DW):

$$C = \frac{A}{V_e} \tag{3}$$

Taking into account the density d and the intracellular compatible solute accumulation A, the volume V_s (mL g^{-1} DW) occupied by the three solutes can be determined as:

$$V_s = \frac{A}{d} * MW \tag{4}$$

where MW is the compatible solute molecular weight.

Glycerol and arabitol densities are 1.26 and 1.40 g L^{-1}, respectively (Larsson et al. 1990); while the density of trehalose density is considered to be similar to that of saccharose, 1.58 g L^{-1} (Weast and Astle 1980–1981).

Results and discussion

Growth on glucose (0.56 M) led to ellipsoidal cells showing a large variability in size (Fig. 1a); the mean volume was 14.3±4.8 µm^3. Cells remained ellipsoidal, but were relatively homogeneous in size when 1.0 mol L^{-1} (Fig. 1b) and 2.0 mol L^{-1} (Fig. 1c) NaCl were added to the culture medium, leading to mean volumes of 8.0±2.4 and 7.1±2.4 µm^3, respectively. In contrast, both morphologies, ellipsoidal and cylindrical, were recorded during growth on 2.2 mol L^{-1} glucose (Fig. 1d), and the mean volumes were 7.6±1.9 and 8.4±2.6 µm^3 respectively, i.e. the volumes were relatively similar despite the different morphologies. The osmotic stress, due to the presence of glucose or NaCl as an osmoticum, therefore resulted in a decrease in the cellular volume (to reduce the exchange surface with the surrounding medium) which was half the volume recorded in the absence of osmotic stress (0.56 mol L^{-1} glucose).

Figure 2a shows an important decrease in the volume accessible to tritiated water with increases in the glucose concentration from 0.01 to 0.1 mol L^{-1}; the decrease was then linear between 0.1 and 2.2 mol L^{-1} glucose (Fig. 2a).

In the presence of NaCl as an osmoticum, A linear decrease of the volume accessible to tritiated water was also observed with increasing NaCl concentrations from 0.5 to 2.0 mol L^{-1} (Fig. 2b). It should be noted that in the absence of NaCl in the culture medium (containing 0.56 mol L^{-1}

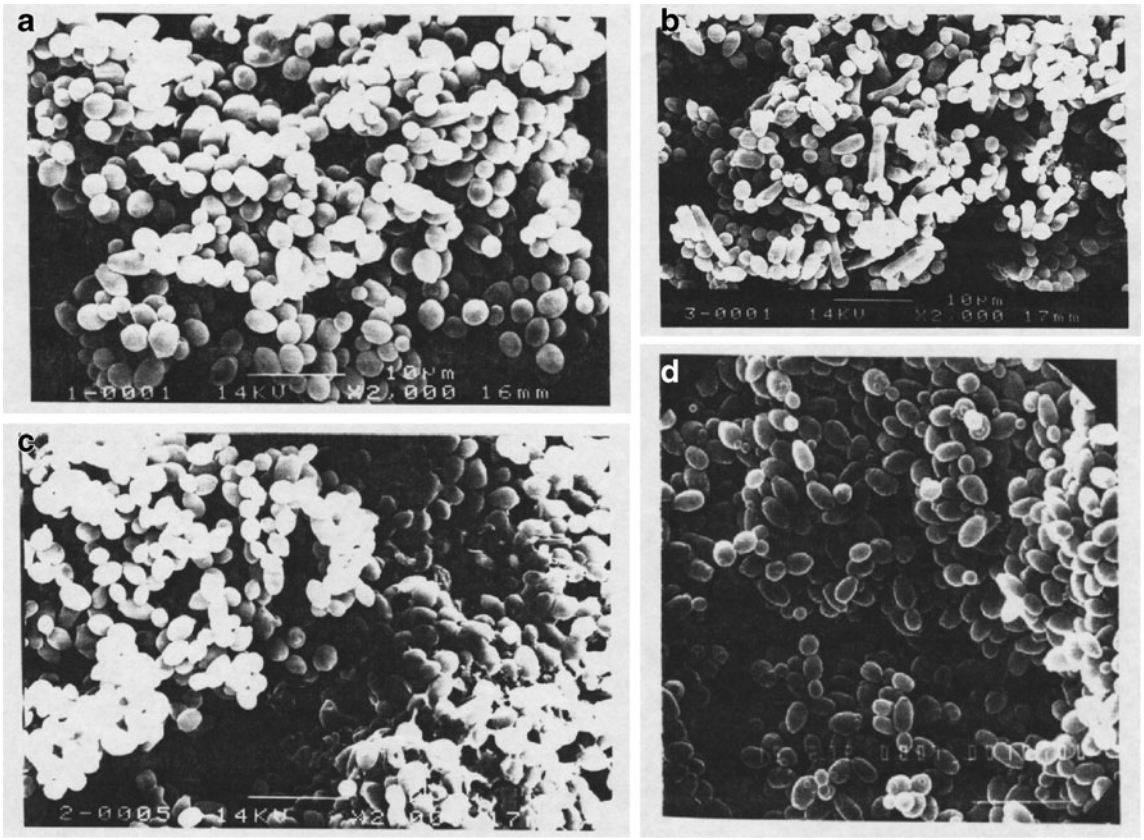

Fig. 1 Scanning electron microscopy (SEM) images of *Hansenula anomala* cells sampled at the end of exponential growth on culture medium containing 0.56 (**a**) or 2.2 (**b**) mol L^{-1} glucose as the carbon substrate and the osmoticum, or 056 mol L^{-1} glucose as the carbon substrate and 1 (**c**) or 2 (**d**) mol L^{-1} NaCl as an osmoticum

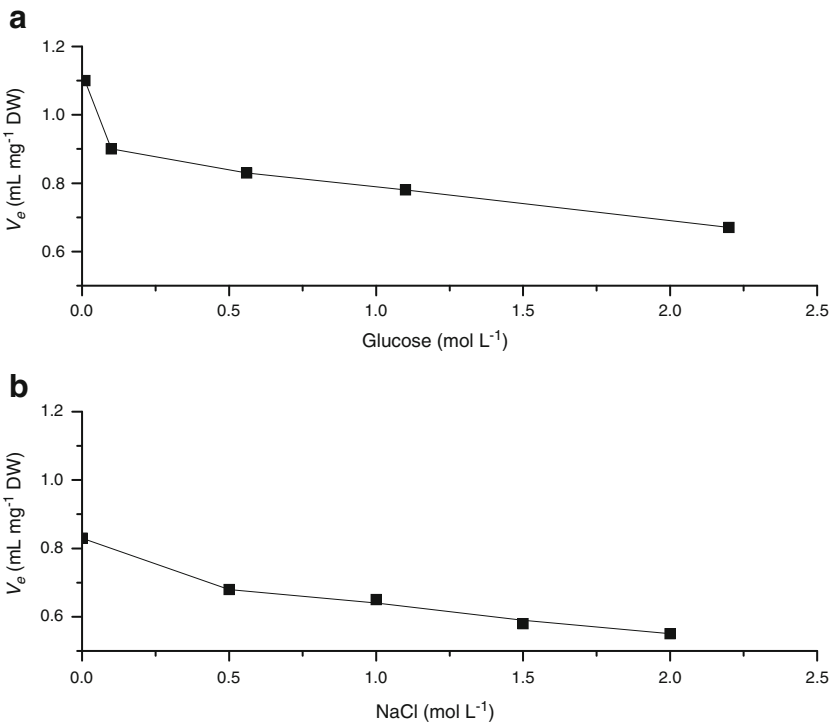

Fig. 2 Evolution of the volume accessible to tritiated water (V_e) of *H. anomala* cells sampled at the end of exponential growth on culture media containing increasing amounts of glucose (**a**) or NaCl (and 0.56 mol L^{-1} glucose) (**b**). *DW* Dry weight

glucose), the accessible volume was 0.82 mL mg^{-1} DW (Fig. 2b), confirming the corresponding value in the case of glucose as an osmoticum, namely, 0.56 mol L^{-1} (Fig. 2a).

Similar effects were recorded for glucose or NaCl as the osmoticum, with the exception of when the osmolarity of the culture medium was low (0.01 mol L^{-1}). Indeed, the decrease in the accessible volume was approximately 0.25 mL mg^{-1} DW (Fig. 2b) with increasing NaCl concentrations from 0 to 2 mol L^{-1} (in the presence of 0.56 mol L^{-1} glucose as a carbon substrate; Fig. 2b), as well as with increasing glucose concentrations from 0.1 to 2.2 mol L^{-1} (Fig. 2a).

The concentration of the compatible solutes accumulated by the yeast cell can be deduced by determining the volume accessible to tritiated water. The objective of our study was to determine whether the accumulation of glycerol, arabitol and trehalose in the cells can balance the internal osmotic potential of cells cultivated in high-osmolarity culture medium.

Figures 3a and b show that the intracellular concentrations of glycerol and arabitol increased with the increased osmotic stress that resulted from increasing concentrations of glucose (Fig. 3a) or NaCl (Fig. 3b), which is in agreement with previous results (Djelal et al. 2005). In response to an osmotic stress, *H. anomala* cells synthesize two polyols, namely, glycerol, the main compatible solute (Nevoigt and Stahl 1997; Tekolo et al. 2010), and arabitol, which has only a secondary role in osmoregulation (Van Eck et al. 1989; Brown 1990; Passoth et al. 2006). In contrast, trehalose accumulation decreased with increasing salt concentrations (Fig. 3b) and when the glucose concentration was more than 0.56 mol L^{-1} (Fig. 3a), which is in agreement with the weak involvement of trehalose in osmoadaptation (Brown et al. 1986; El Mokadem et al. 1986). The accumulation of trehalose in cells has been shown to be a specific response to oxygen limitation (Fredlund et al. 2004; Walker 2011). The greater amounts of intracellular glycerol and arabitol that accumulated with increasing concentrations of of NaCl in the culture medium (Fig. 3b), in comparison to increased glucose concentrations (Fig. 3a), suggest that NaCl has a higher water stress effect on *H. anomala* than glucose. Indeed, the minimal water activity for *H. anomala* growth has been found to be lower in the presence of glucose as an osmoticum, compared to NaCl, namely, 0.865 and 0.875, respectively (Van Eck et al. 1989). This greater tolerance to glucose than to NaCl corresponds to the general behaviour of yeasts (Van Eck 1988).

The contribution of each compatible solute to the osmotic potential (Table 1) followed their level of accumulation in cells (Fig. 3). Table 1 shows that in the case of a low-osmolarity culture medium, the sum of the contributions of glycerol, arabitol and trehalose adjusted to the osmotic potential of cells. In contrast, this was not the case for increasing medium osmolarity, since the differential between the sum of the solute contributions and the osmotic pressure of the initial culture medium was clearly negative for all amounts of NaCl added to the culture medium (at least −0.3 MPa) and for 2.2 mol L^{-1} glucose (−0.2 MPa) (Table 1). For similar osmoticum concentrations, the

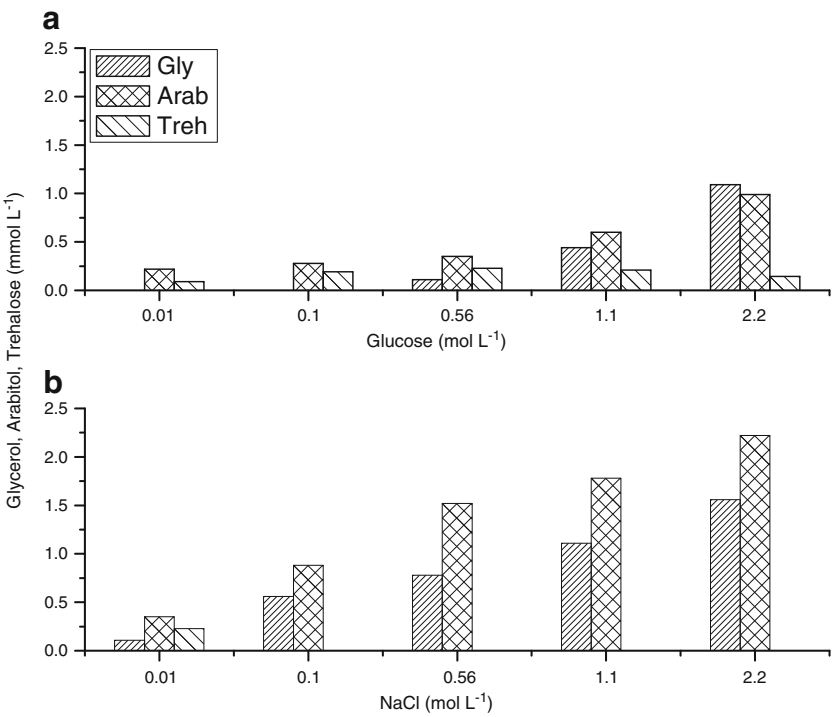

Fig. 3 Intracellular concentrations of glycerol (*Gly*), arabitol (*Arab*) and trehalose (*Treh*) accumulated in *H. anomala* cells at the end of the growth phase for a range of osmoticum concentrations added to the culture medium: **a** glucose, **b** NaCl (in the presence of 0.56 mol L^{-1} glucose in the medium)

Table 1 Effect of the osmoticum on the osmotic potential

Osmoticum (mol L^{-1})	Dry weight (g L^{-1})	Osmotic potential					Difference[c] (−MPa)
		Medium[a](− MPa)	Compatible solutes (−MPa)				
			Glycerol[b]	Arabitol[b]	Trehalose[b]	Sum	
Glucose							
0.01	1.0	0.0247	0	0.544	0.06	0.604	0.579
0.1	3.0	0.2476	0	0.693	0.13	0.823	0.5754
0.56	3.1	1.386	0.272	0.866	0.16	1.298	−0.088
1.1	3.3	2.723	1.089	1.485	0.15	2.724	0.001
2.2	3.4	5.447	2.698	2.451	0.10	5.249	−0.198
NaCl							
0.0	3.1	1.386	0.269	0.866	0.160	1.295	−0.091
0.5	3.6	3.862	1.386	2.178	-	3.564	−0.298
1.0	2.6	6.338	1.93	3.76	-	5.69	−0.648
1.5	2.9	8.814	2.748	4.407	-	7.155	−1.658
2.0	1.9	11.29	3.862	5.496	-	9.358	−1.932

[a] Initial culture medium

[b] To determine the osmotic potential due to the compatible solutes, the Van't Hoff relation was considered: $\pi = I \times C \times R \times T \times 1000$ where π is the osmotic pressure (Pa), i is the Van't Hoff coefficient (i=2 for NaCl owing to its total dissociation in solution and i=1 for glucose, glycerol, arabitol and trehalose), C is the molar intracellular solute concentrations deduced from Fig. 3, R is the universal gas constant (8.31 J mol^{-1} K^{-1}), T is the room temperature (298 K) and the factor 1000 allows the conversion from litre to m^3 to obtain the osmotic pressure π in Pa

[c] Difference between the contributions of the three solutes and the initial culture medium

osmolarity of the medium was higher with NaCl as the osmoticum, owing to its total dissociation. However, this phenomenon cannot account for the more negative differential values recorded in the case of NaCl as an osmoticum, since our comparison of cultures in media with a similar osmolarity clearly showed the higher stress effect of NaCl (Table 1), confirming the higher tolerance of the cells to glucose than to NaCl. This higher tolerance was also confirmed by the decrease in biomass concentration that was only observed in the presence of NaCl as the osmoticum (Table 1). A decrease in final biomass concentrations was recorded from 0.5 mol L^{-1} NaCl upwards; in comparison at a similar medium osmolarity due to a high glucose concentration (2.2 mol L^{-1}), no decrease in the final biomass concentration was recorded (Table 1).

As expected, for increasing amounts of the osmoticum, glucose or NaCl, the volume accessible to tritiated water decreased, while the volume occupied by the compatible solutes increased (Table 2) owing to the increase in compatible solute concentrations (Fig. 3). However, the sum of the two volumes decreased with increasing concentrations of the osmoticum, glucose or NaCl (Table 2), showing that the volume occupied by the compatible solutes could not counterbalance the decrease in cell volume with increasing water stress.

Compared to the values recorded with glucose as an osmoticum, for any given salt concentration, a lower cell volume was accessible to tritiated water and a higher volume was occupied by the compatible solutes (Table 2),

Table 2 Effect of the osmoticum on the cell volume accessible to tritiated water (V_e) and the volume occupied by the main compatible solutes, glycerol, arabitol and trehalose (V_s)

Osmoticum (mol L^{-1})	Volumes (µL mg^{-1} DW)			
	V_e[a]	V_s[b]	$V_e + V_s$	$\frac{V_s}{V_e+V_s}$ (%)
Glucose				
0.01	1.10±0.06	0.044	1.14	3.9
0.1	0.90±0.03	0.063	0.96	6.6
0.56	0.82±0.03	0.074	0.89	8.3
1.1	0.76±0.06	0.115	0.87	12.6
2.2	0.66±0.04	0.130	0.79	16.5
NaCl				
0.0	0.82±0.03	0.07	0.89	8.3
0.5	0.68±0.02	0.15	0.83	18.1
1.0	0.64±0.04	0.16	0.80	20.0
1.5	0.58±0.03	0.17	0.75	22.7
2.0	0.54±0.03	0.20	0.74	27.0

DW, Dry weight

[a] Measured values were the average of ten measurements

[b] Calculated values deduced from the compatible solute concentrations given in Fig. 3

thereby confirming the higher salt effect relative to the glucose effect. The consequence of the above was that the contribution of the compatible solutes to the total volume increased with increasing osmoticum concentration, and this increase was more pronounced in the presence of salt as an osmoticum.

Conclusions

With osmotic stress resulting from high glucose or NaCl concentrations, there was no noticeable change in cell morphology, and the cells remained ellipsoidal. However, there was a decrease in the cell volume with increasing water stress, since the decrease in the volume accessible to tritiated water was not counterbalanced by the contribution of the compatible solutes to the cell volume. The higher levels of intracellular glycerol and arabitol that accumulated with increasing amounts of NaCl in the culture medium, relative to glucose concentrations, suggest that NaCl has a higher water stress effect on *H. anomala* than glucose. Indeed, the minimal water activity for *H. anomala* growth was lower in the presence of glucose as an osmoticum than in the presence of NaCl (0.865 and 0.875 respectively), which is in agreement with the general yeast behaviour.

Acknowledgements We wish to thank Dr. Annette Bourgeois for reviewing the English of the manuscript.

References

Adler L, Gustafsson L (1980) Polyhydric alcohol production and intra-cellular amino-acid pool in relation to halotoleranc of the yeast *Debaromyces hansenii*. Arch Microbiol 124:123–130

Blomberg A (2000) Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. FEMS Microbiol Lett 182:1–8

Brown AD (1990) Microbial water stress physiology principles and perspectives. John Wiley and Sons, New York

Brown AD, Edgley M (1980) Osmoregulation in yeast. In: Rawis DW, Valentine RC, Hollaender A (eds) The genetic engineering of osmoregulation. Plenum, New York, pp 75–90

Brown AD, McKensie KF, Singh KK (1986) Selected aspects of microbial osmoregulation. FEMS Microbiol Rev 39:31–36

Djelal H, Amrane A, Lahrer F, Martin G (2005) Effect of medium osmolarity on the bioproduction of glycerol and ethanol by *Hansenula anomala* growing on glucose and ammonium. Appl Microbiol Biotechnol 69:341–349

Djelal H, Larher F, Martin G, Amrane A (2006) Effect of the dissolved oxygen on the bioproduction of glycerol and ethanol by Hansenula anomala growing on glucose and ammonium. J Biotechnol 125:95–103

El Mokadem MT, Mahmoud MI, Kairalla ZH (1986) The effect of growth medium on the levels of trehalose and glycogen content of some yeasts. Egypt J Microbiol 21:103–109

Fredlund E, Broberg A, Boysen ME, Kenne L, Schnürer J (2004) Metabolite profiles of the biocontrol yeast *Pichia anomala* J121 grown under oxygen limitation. Appl Microbiol Biotechnol 64:403–409

Gustafsson L, Norkrans B (1976) On the mechanism of salt tolerance - Production of glycerol and heat during growth of *Debaromyces hansenii*. Arch Microbiol 110:177–183

Hohmann S, Krantz M, Nordiander B (2007) Yeast osmoregulation. Methods Enzymol 428:29–45

Katz M, Avron M (1985) Determination of intracellular osmotic volume and sodium concentration in *Dunaliella*. Plant Physiol 78:817–820

Larsson C, Morales C, Gustafsson L, Adler L (1990) Osmoregulation of the salt-tolerant yeast *Debaromyces hansenii* grown in chemostat at different salinities. J Bacteriol 172:1769–1774

Meikle AJ, Reed RH, Gadd GM (1988) Osmotic adjustment and the accumulation of organic solutes in whole cells and protoplasts of *Saccharomyces cerevisiae*. J Gen Microbiol 134:3049–3060

Morris GJ, Winters L, Coulson GE, Clarke KJ (1986) Effect of somotic stress on the ultrastructure and viability of the yeast *Saccharomyces cerevisiae*. J Gen Microbiol 132:2023–2034

Nevoigt E, Stahl U (1997) Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 21:231–241

Passoth V, Fredlund E, del Druvefors E, Schnürer J (2006) Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res 6:3–13

Passoth V, Olstorpe M, Schnürer J (2011) Past, present and future research directions with *Pichia anomala*. Antonie Van Leeuwenhoek 99:121–125

Reed RH, Chudek JA, Foster R, Gadd GM (1987) Osmotic significance of glycerol accumulation in exponentially growing yeasts. Appl Environ Microbiol 53:2119–2123

Rose D (1975) Physical responses of yeast cells to osmotic shock. J Appl Bacteriol 38:169–175

Stock JB, Rauch B, Roseman S (1977) Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J Biol Chem 252:7850–7861

Tekolo OM, McKenzie J, Botha A, Prior BA (2010) The osmotic stress tolerance of basidiomycetous yeasts. FEMS Yeast Res 10:482–491

Van Eck JH (1988) Water relations of polyhydroxy alcohol accumulation by yeasts. Magister Scientiae Thesis. The Orange Free State University, Bloemfontein

Van Eck JH, Prior BA, Brandt EV (1989) Accumulation of polyhydroxy alcohols by *Hansenula anomala* in response to water stress. J Gen Microbiol 135:3505–3513

Walker GM (2011) *Pichia anomala*: cell physiology and biotechnology relative to other yeasts. Antonie Van Leeuwenhoek 99:25–34

Weast RC, Astle MJ (1980–1981) Handbook of chemistry and physics. 61th edn. CRC Press, Boca Raton

Wickerham LJ (1951) Taxonomy of yeasts. United States Department of Agriculture, Washington D.C.