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Comparative analysis of trehalose production by *Debaryomyces hansenii* and *Saccharomyces cerevisiae* under saline stress

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Abstract The comparative analysis of growth, intracellular content of Na^+ and K^+, and the production of trehalose in the halophilic *Debaryomyces hansenii* and *Saccharomyces cerevisiae* were determined under saline stress. The yeast species were studied based on their ability to grow in the absence or presence of 0.6 or 1.0 M NaCl and KCl. *D. hansenii* strains grew better and accumulated more Na^+ than *S. cerevisiae* under saline stress (0.6 and 1.0 M of NaCl), compared to *S. cerevisiae* strains under similar conditions. By two methods, we found that *D. hansenii* showed a higher production of trehalose, compared to *S. cerevisiae*; *S. cerevisiae* active dry yeast contained more trehalose than a regular commercial strain (*S. cerevisiae* La Azteca) under all conditions, except when the cells were grown in the presence of 1.0 M NaCl. In our experiments, it was found that *D. hansenii* accumulates more glycerol than trehalose under saline stress (2.0 and 3.0 M salts). However, under moderate NaCl stress, the cells accumulated more trehalose than glycerol. We suggest that the elevated production of trehalose in *D. hansenii* plays a role as reserve carbohydrate, as reported for other microorganisms.

Keywords *Debaryomyces hansenii* · Glycerol · Halophile · *Saccharomyces cerevisiae* · Saline stress · Trehalose

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Introduction

It has been previously shown that the halophile yeast *Debaryomyces hansenii* accumulates high levels of intracellular Na^+ and K^+ when grown at high salinity (Norkrans and Kylin 1969), and that glycerol accumulates intracellularly proportionally to increasing extracellular NaCl concentrations (Gustafsson and Norkrans 1976). It was also reported that in *D. hansenii*, the intracellular levels of polyols are markedly enhanced by high salinity, the dominant solutes being glycerol in log phase cells and arabinitol in stationary phase cells (Adler and Gustafsson 1980).

Two different fundamental adaptation strategies exist, allowing certain microorganisms to bear osmotic stress due to the presence of a high concentration of salt, as described by González-Hernández and Peña (2002): (1) the cells maintain high intracellular concentrations of salt, osmotic or at least equivalent to the external concentration of salt (salt-in strategy), and intracellular systems must adapt to the presence of high concentrations of salt; and (2) the cells maintain lower concentrations of salts in their cytoplasm (compatible-solute strategy). The osmotic pressure of the medium is balanced by compatible solutes, and no special adaptation of the intracellular systems is required (Bayley and Morton 1978).

Under osmotic stress, microorganisms must be able to restore their volume and turgor pressure by increasing the internal osmolarity, and many microorganisms respond to such environmental change by accumulating low-molecular-weight organic solutes (LeRudulier et al. 1984; Higgins et al. 1987). The transfer of growing cells of the salt-tolerant yeast *D. hansenii* to media of higher salinity resulted in an increased production and intracellular accumulation of glycerol, proportional to the magnitude of the salinity shift (André et al. 1988).

Jovall et al. (1990) reported the accumulation of organic solutes in *D. hansenii* after transfer to media of increased salinity. Cells were incubated with labeled

glucose, and the intracellular metabolic processes were assessed by NMR spectroscopy of intact cells or cellular extracts. The analysis of 17 yeast strains by ^{13}C-NMR spectroscopy has been reported, confirming the significance of glycerol as the sole osmoregulatory solute under salt-stressed conditions (Meikle et al. 1991). Khroustalyova et al. (2001), reported that the yeast *D. hansenii* is highly resistant to dehydration stress, and this tolerance was more pronounced for cells taken from the exponential growth phase than from the stationary phase; the growth of *D. hansenii* in media containing 10% (w/v) NaCl resulted in an additional increase in cellular resistance to dehydration, which was most marked for stationary-phase cells.

Iwahashi et al. (2000), reported evidence of contribution of neutral trehalase in barotolerance (resistance to hydrostatic pressure), an effect mediated by neutral trehalase codified by *NTH1*. The deletion of *NTH1* decreased barotolerance, and its reintroduction increased barotolerance. Furthermore, induction of neutral trehalase activity under recovery conditions significantly increased barotolerance. Thus, neutral trehalase contributed to barotolerance, especially during recovery conditions.

Trehalose is a ubiquitous disaccharide in the biosphere; it has been isolated and characterized from a large variety of both prokaryotic and eukaryotic organisms, ranging from bacteria to plants and mammals (Elbein 1974; Thevelein 1984; Thevelein and Hohmann 1995; Strom and Kaasen 1993). Varieties of functions have been proposed for trehalose, which depend on the specific biological system analyzed. In some yeast and filamentous fungi, large amounts of trehalose are stored both as a reserve carbohydrate and as protector against stress challenge to cells.

Since *D. hansenii* is one of the salt-resistant species of yeast, its study could contribute to a better understanding of the phenomenon of halotolerance and osmoregulation in halophilic organisms. The present work is aimed to make a comparative analysis of growth, intracellular content of Na^+ and K^+, trehalose production, and attempt to understand the role of trehalose in this halophilic yeast. In this study, we used three different strains of *D. hansenii* (Y7426, J26, and CBS1793) and two commercial strains of *Saccharomyces cerevisiae* [La Azteca and active dry yeast (ADY)]. The experiments were carried out under different saline stress conditions.

Materials and methods

Yeasts strains, media, and culture conditions

Debaryomyces hansenii strain Y7426 was obtained from the US Department of Agriculture, Peoria, Ill., USA; *D. hansenii* strain J26 and *D. hansenii* strain CBS1793 were kindly supplied by L. Adler and H. Sychrová, respectively. *Saccharomyces cerevisiae* Baker's yeast was obtained locally (La Azteca, S.A.); *S. cerevisiae* ADY was obtained from Sigma (St. Louis, Mo., USA, cat. no. YSC-2). The rehydration procedure was that reported by Peña et al. (1992). The yeasts were kept in YPD plates containing 1% yeast extract, 1% bactopeptone, and 2% glucose, plus 2% bactoagar. Liquid cultures were started from a 100-ml inoculum of cells grown in YPD, with or without 0.6, 1.0, 2.0, or 3.0 M of either KCl or NaCl. Inocules were grown at 30°C for 24 h in a gyrotory shaker at 250 rpm. Cultures (0.5 l) were then grown under similar conditions, but for 36 h. The cultured cells were collected by centrifugation and washed twice with water. The final pellets of cells grown in YPD (C), with the addition of 0.6, 1.0, 2.0, and 3.0 M NaCl (Na06, Na1, Na2, and Na3, respectively) or with 0.6, 1.0, 2.0, and 3.0 M KCl (K06, K1, K2, and K3, respectively), were finally resuspended in water to a concentration of 0.5 g/ml (w/v) and kept on ice to be used the same day. Some experiments were also carried out with cells starved by aeration in water for 14 h.

Determination of intracellular Na^+ and K^+ content

To measure the internal Na^+ and K^+ content, cells grown in different conditions were collected and washed twice with water by centrifugation, and then suspended in water to a concentration of 0.5 g/ml and kept on ice; this procedure removed the Na^+ or K^+ attached to the outside of the cells. The cells (50 mg, wet weight) were disrupted by incubation with 200 µM cetyltrimethylammonium bromide (CTAB) for 15 min at room temperature. The suspension was centrifuged, and the cation concentration in the supernatant was determined with a Zeiss PF5 flame photometer. The concentrations of cations were calculated after measuring the internal value of water under the different conditions, determined from the distribution of radioactive isotopes ^{14}C-sucrose and ^3H_2O in the supernatant obtained from 500 µl of cells (0.5 g/ml) and assuming the uniform distribution of the latter within the cell (González-Hernández et al. 2004). In *S. cerevisiae*, internal volume was 47% of the wet weight of the cells (Peña et al. 1967).

Growth

Growth was followed by the absorbance changes at 600 nm with a Shimadzu spectrophotometer, model UV-160A.

Extraction of samples for trehalose and glycerol determination

Samples (1 ml) were extracted by boiling the cell suspensions at 92°C in a water bath for 15 min. After centrifugation the supernatant was frozen until analysis. Trehalose and glycerol were measured in adequate

dilutions of the supernatant (Kienle et al. 1993; Adler and Gustafsson 1980, respectively).

Glycerol determination

Glycerol was phosphorylated to glycerol-3-phosphate by ATP in the presence of glycerol kinase. Glycerol-3-phosphate was then converted to dihydroxyacetone phosphate by glycerophosphate dehydrogenase, leading to the formation of NADH. The increase in NADH concentration was recorded with a Shimadzu spectrophotometer, model UV-160A at 340 nm (Wieland 1965).

Thin-layer chromatography method

Standard 0.1 M solutions of pure trehalose, galactose, glucose, mannitol, sorbitol, maltose, sucrose, raffinose, and glycerol were prepared. The crystals obtained for the NMR spectra of the samples obtained from *D. hansenii* Y7426, cultivated in the absence and in the presence of NaCl or KCl, were used to prepare a 0.1-M stock solution. From the extracts and standards prepared, 10 µl was taken and diluted with 40 µl pyridine; 1 µl of this mix was taken and spotted onto Silica Gel 60 F_{254} plates (Merck, Darmstadt, Germany) and developed in a closed chamber with 1-butanol:isopropanol:water (10:5:4 vol/vol) as the mobile phase. The spots were detected by spraying the plates with vanillin, 5 mg/ml in concentrated H_2SO_4 and 5% ethanol, and heating at 130°C (modified from Sue and Pringle 1980).

Assay of trehalose with acid trehalase—uncoupled assay

The extracts obtained by boiling the cell suspensions at 92°C in a water bath for 15 min were used. Extracts (100 µl) plus 25 µl trehalase were incubated overnight (14 h) at 40°C. The resulting glucose was assayed with glucose oxidase by adding to the spectrophotometer cell (2.0 ml final volume), the following reagents: 1% *o*-dianisidine, prepared by diluting in 0.1 M phosphate buffer pH 6.0; digested trehalose sample; peroxidase, 200 µg/ml in distilled water (1.5 U/ml of the assay mixture); and glucose oxidase (1 mg/ml in distilled water, to obtain 21 U/ml in 2 ml of the assay mixture). Absorbance changes were measured in the spectrophotometer at 460 nm and 25°C and compared to a glucose standard curve (modified from that of Araujo et al. 1989).

NMR spectroscopy

Stationary-phase cells (36 h) were extracted by boiling in a water bath for 15 min and centrifuging to obtain the supernatant; carbohydrates were extracted immediately with 80% (v/v) ethanol (50 ml). After overnight extraction, ethanol was removed by rotary evaporation (Büchi Heating Bath B-490), and the samples were then dried in a desiccator for at least 48 h. Dried samples were resuspended in D_2O (Aldrich Chemical) and analyzed using NMR spectroscopy. All spectra were obtained with a Bruker spectrometer model; the samples of ^{13}C-NMR spectra were obtained at 300 Mhz. The trehalose peak was identified against that of the pure substance.

Viability determination

Liquid cultures were started from a 100-ml inoculum of cells grown in YPD, with variable concentrations of either KCl or NaCl, and then grown at 30°C for 24 h in a gyrotory shaker at 250 rpm. Immediately after, samples were taken, and successive tenfold dilutions were prepared in sterile water to count the cells (*D. hansenii* Y7426). Then, 150 cells of each culture were spread in duplicate plates containing YPD media without salt, or with 0.6, 1.0, 2.0, or 3.0 M of NaCl or KCl. The plates were incubated for 36 h (YPD, 0.6 and 1.0 M NaCl or KCl) and 200 h (2.0 and 3.0 M of NaCl or KCl) at 30°C. After this time, the colonies were counted to determine viability.

Reproducibility

All assays were repeated at least three times, and the data reported are mean values; however, the number of independent experiments and replicates is mentioned whenever relevant.

Results

Figure 1a shows the influence of NaCl and KCl on the growth of *Debaryomyces hansenii* Y7426 in YPD without salt (C) or in the presence of 0.6 M (Na06 or K06), and 1.0 M (Na1 or K1) of NaCl or KCl. An increased growth rate was observed in the cells grown in Na06 or K06, compared to other concentrations; however, growth rate was similar to the control of 1.0 M of either NaCl or KCl. *D. hansenii* J26 (Fig. 1b) showed a slight reduction in growth at all concentrations of cells, which was more marked after 20 h in Na1 cells. The *D. hansenii* CBS1793 strain grew faster at 0.6 M than all other cells conditions; in this strain the yeast growth order is: K06 > Na06 > K1 > C > Na1. In Fig. 1d, e, it is shown that *Saccharomyces cerevisiae* La Azteca and ADY showed similar growth behavior under all experimental conditions; however, the largest decrease of growth rate was observed with NaCl at all concentrations, particularly at 1.0 M. Table 1 shows the doubling times; 0.6 M salt produced the highest growth rate for *D. hansenii* Y7426, in agreement with data obtained by González-Hernández et al. (2004), while J26 and

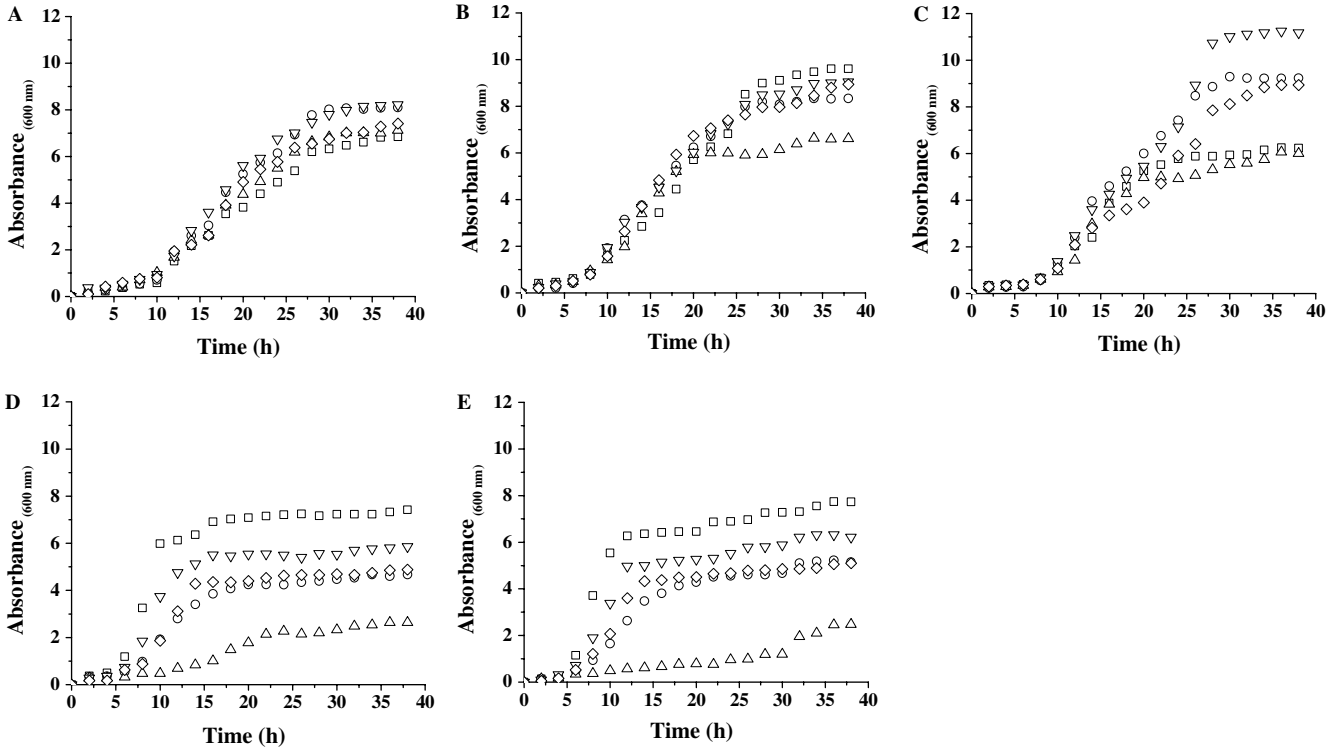

Fig. 1 Growth of *Debaryomyces hansenii* **a** strain Y7426, **b** strain J26, **c** strain CBS1793 and *Saccharomyces cerevisiae* **d** La Azteca, **e** and active dry yeast, all under different saline stress conditions. *Open square* Without salt (C), *open circle* 0.6 M NaCl (Na06), *open triangle* 1.0 M NaCl (Na1), *open inverted triangle* 0.6 M KCl (K06), *open diamond* 1.0 M KCl (K1). Results are the means of three experiments

CBS1793 practically did not show differences. With *S. cerevisiae* La Azteca and ADY, we found an increase of doubling time in the cells cultivated in the presence of 0.6 and 1.0 M of each salt (Table 1).

Table 2 shows the content of Na^+ and K^+ of the different strains from *D. hansenii* and *S. cerevisiae* analyzed in this work under the different experimental conditions; the internal concentrations of Na^+ and K^+ in *D. hansenii* Y7426 under different experimental conditions (Table 2) show that C cells contained only a small amount of Na^+, compared to K^+. Na06 cells accumulated high concentrations of Na^+, with moderate concentrations of K^+. At both NaCl concentrations in the growth medium, the internal Na^+ concentration was higher than that of K^+. The K06 and K1 cells contained a very low concentration of Na^+, while K^+ concentration rose to around 200 mM. In the cells grown in the presence of NaCl (Na06 and Na1), the *D. hansenii* Y7426 strain accumulated almost twice the concentration of Na^+ in comparison with the J26 and CBS1793 strains (Table 2). *D. hansenii* accumulated K^+ concentrations varying from 180–230 mM, except for the Y7426 strain, which, when cultivated in the presence of NaCl, accumulated less and less K^+, depending on the increasing concentrations of Na^+ in the medium [these data collected for the Y7426 strain agree with the data obtained by González-Hernández et al. (2004)]. The commercial strain of *S. cerevisiae* in Na06 also accumulated significantly more Na^+ than ADY. When grown in

Table 1 Doubling time of *Debaryomyces hansenii* and *Saccharomyces cerevisiae* grown in different media

Medium	Doubling time (h)[a]				
	D. hansenii strain Y7426	*D. hansenii* strain J26	*D. hansenii* strain CBS1793	*S. cerevisiae* La Azteca	*S. cerevisiae* ADY[b]
C	3.2	2.28	2.5	0.76	0.93
Na06[c]	2.75	2.44	2.13	1.89	2.5
Na1	3.17	2.22	2.32	3.33	4.45
K06	2.56	2.37	1.95	1.3	1.68
K1	3.7	1.88	2.63	1.37	1.95

[a]Determined from the exponential phase of the curves. Cells were grown as described under "Materials and methods." Results are the means of three experiments

[b]*ADY* Active dry yeast

[c]*Na06* 0.6 M NaCl, *Na1* 1.0 M NaCl, *K06* 0.6 M KCl, *K1* 1.0 M KCl

Table 2 Intracellular concentrations of Na^+ and K^+ of cells from different yeast strains grown in the absence or presence of different concentrations of NaCl or KCl

Salt concentration	*D. hansenii*[a]			*S. cerevisiae*[a]	
	Y7426	J26	CBS1793	La Azteca	ADY
Na^+ (mM)					
C	10.2 ± 1.7	9.0 ± 0.7	10.5 ± 2.6	9.5 ± 0.3	2.8 ± 0.6
Na06	186.3 ± 14.2	100.6 ± 4.9	113.9 ± 7.5	70.5 ± 1.6	48.2 ± 0.5
Na1	211.7 ± 18.7	134.2 ± 7.1	118.1 ± 18.4	167.6 ± 7.2	135.6 ± 12.0
K06	3.1 ± 1.0	9.3 ± 0.8	6.8 ± 3.0	6.1 ± 3.1	3.0 ± 0.3
K1	2.4 ± 1.1	5.0 ± 0.3	23.3 ± 20.8	8.7 ± 1.2	1.3 ± 0.1
K^+ (mM)					
C	180.3 ± 13.3	196.4 ± 6.6	212.1 ± 6.0	287.3 ± 8.4	132.0 ± 2.6
Na06	111.2 ± 18.7	225.4 ± 14.4	236.7 ± 8.9	198.3 ± 15.5	121.0 ± 3.4
Na1	61.4 ± 7.1	184.2 ± 14.6	193.2 ± 31.9	108.5 ± 13.9	33.0 ± 2.0
K06	218.7 ± 27.4	230.6 ± 10.9	233.5 ± 11.1	216.1 ± 7.2	124.3 ± 3.7
K1	198.9 ± 20.6	236.8 ± 21.3	208.7 ± 14.2	225.9 ± 25.2	123.3 ± 2.5

[a]After growth, centrifugation, and twice washing with water, the cells (50 mg) grown for 36 h were disrupted by incubation with 200 μM CTAB for 15 min at room temperature; the suspension was centrifuged, and adequate dilutions of the supernatant were used to measure Na^+ and K^+ in the flame photometer. Results are the means of three experiments

Table 3 Effect of NaCl and KCl on trehalose content of *D. hansenii* and *S. cerevisiae* cells at the stationary phase without starvation

	D. hansenii[a]			*S. cerevisiae*[a]	
	Y7426	J26	CBS1793	La Azteca	ADY
Trehalose (μM)					
C	424.5 ± 4.9	427.1 ± 4.7	426.2 ± 5.4	40.0 ± 0.7	95.9 ± 4.9
Na06	392.7 ± 3.7	414.7 ± 2.6	422.5 ± 2.6	64.9 ± 1.6	326.9 ± 4.4
Na1	273.1 ± 4.0	432.7 ± 4.4	427.2 ± 1.5	17.1 ± 0.5	15.9 ± 0.8
K06	424.4 ± 5.5	413.2 ± 1.5	422.4 ± 2.2	50.3 ± 2.2	213.5 ± 1.5
K1	301.3 ± 21.3	426.6 ± 5.2	419.2 ± 2.9	7.9 ± 0.6	162.7 ± 2.1

[a]After growth, the cells were disrupted by boiling the cell suspensions at 92°C in a water bath for 15 min. After centrifugation the supernatant was frozen until analysis. In adequate dilutions of the supernatant, trehalose was measured as described in "Materials and methods"

the presence of 1 M of NaCl, the La Azteca strain also accumulated more than twice the amount of Na^+ and significantly more than the ADY strain. *S. cerevisiae* La Azteca accumulated the highest concentrations of K^+, which also decreased as the concentration of Na^+ in the medium was increased. A similar behavior was observed with the ADY strain, which, however, accumulated less K^+ under all conditions tested, even at the highest concentrations of K^+ in the medium.

Table 3 shows the production of trehalose for the different strains. The *D. hansenii* Y7426 strain accumulated around 425 μM when grown in YPD medium, which decreased as the NaCl concentration in the medium was increased, and at 1.0 M KCl. With the two other strains of *D. hansenii* (J26 and CBS1793), no changes in the content of trehalose were observed when the cells were grown with different NaCl or KCl concentrations. Both *S. cerevisiae* strains, when grown in the presence of salt, accumulated more trehalose at 0.6 M of NaCl or KCl, and at much less at 1.0 M of both salts. It is also interesting that ADY, following a similar pattern respect to the response to salt, always showed a much higher level of trehalose under all conditions, except for the high concentrations of both NaCl and KCl, and that at 1.0 M KCl, the decrease of the trehalose content was much lower than that of the other commercial strain.

Figure 2a shows the resonance spectrum of standard trehalose (Sigma) and Fig. 2b–d, the spectra of the cellular extracts (*D. hansenii* Y7426 strain) obtained at different experimental conditions (C, Na06, and K06 cells). This strain was employed for further studies, because we evaluated some metabolites and glycolytic enzymes, suggesting that probably this carbohydrate might play an important role in that metabolic route (data not published). Figure 2 shows the peak positions (ppm): $C_1 = 93.86$, $C_2 = 73.2$, $C_3 = 72.79$, $C_4 = 71.7$, $C_5 = 70.36$, and $C_6 = 61.23$, which were practically identical for the pure commercial substance and that obtained from the cell extracts. The same samples used to obtain the spectra were also analyzed by thin-layer chromatography (TLC) (Fig. 3); from both results, we concluded that obtained samples were pure trehalose.

Figure 3a (*a–i*) shows the spots obtained with the different pure sugars used in the TLC: trehalose, galactose, glucose, mannitol, sorbitol, maltose, sucrose,

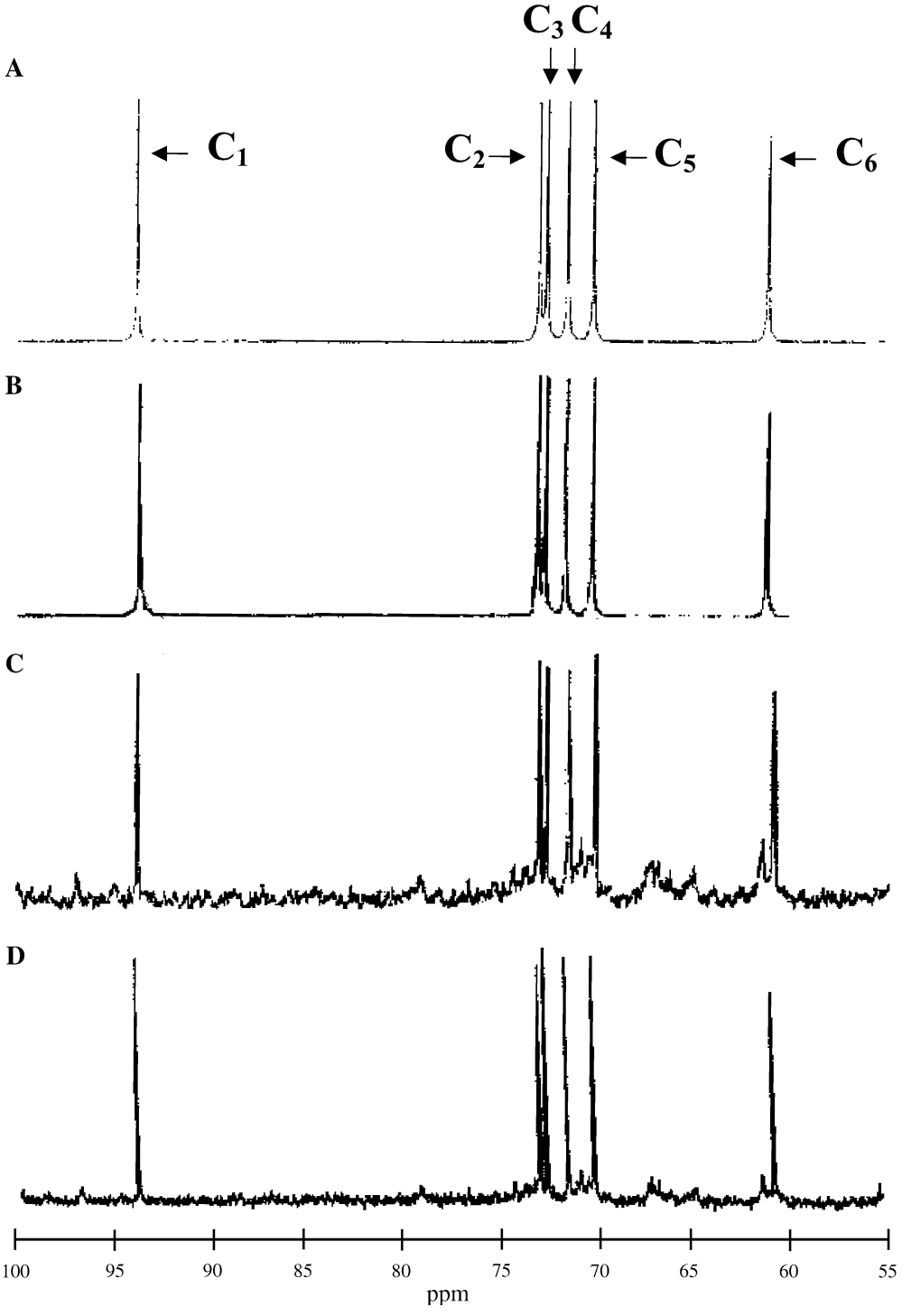

Fig. 2 Natural abundance ^{13}C-NMR spectra of dried samples. Trehalose control (**a**) and cells extracts at the stationary phase of *D. hansenii* Y7426 (**b**–**d**) grown without and with salt stress. Sigma trehalose reagent (**a**), C cells (**b**), Na06 cells (**c**), K06 cells (**d**). The 100.0–55.0 ppm region of the spectrum is shown. Trehalose peaks: $C_1 = 93.86$, $C_2 = 73.2$, $C_3 = 72.79$, $C_4 = 71.7$, $C_5 = 70.36$, and $C_6 = 61.23$

raffinose, and trehalose, respectively. Figure 3b shows the spots of the obtained trehalose of *D. hansenii* Y7426 (Fig. 3b, *c*–*k*) and *S. cerevisiae* (Fig. 3b) cultivated under different experimental conditions: control trehalose, *S. cerevisiae* La Azteca cultivated in YPD, C, Na06, Na1, Na2, Na3, K06, K1, K2, K3, and control trehalose [Fig. 3b (*a*–*l*, respectively)].

Figure 4 shows the glycerol content of *S. cerevisiae* La Azteca grown in YPD (Fig. 4, *a*), and *D. hansenii* Y7426 (Fig. 4, *b*–*j*) strain determined under saline stress. A 50-μM basal content was found, which increased to 100 μM in the presence of high concentrations of salt in the growth medium (2.0 and 3.0 M of NaCl or KCl, respectively). The same graph presents the production of trehalose under the same conditions of growth; contrary to glycerol, the production of trehalose decreased with the increasing concentrations of salt in the medium.

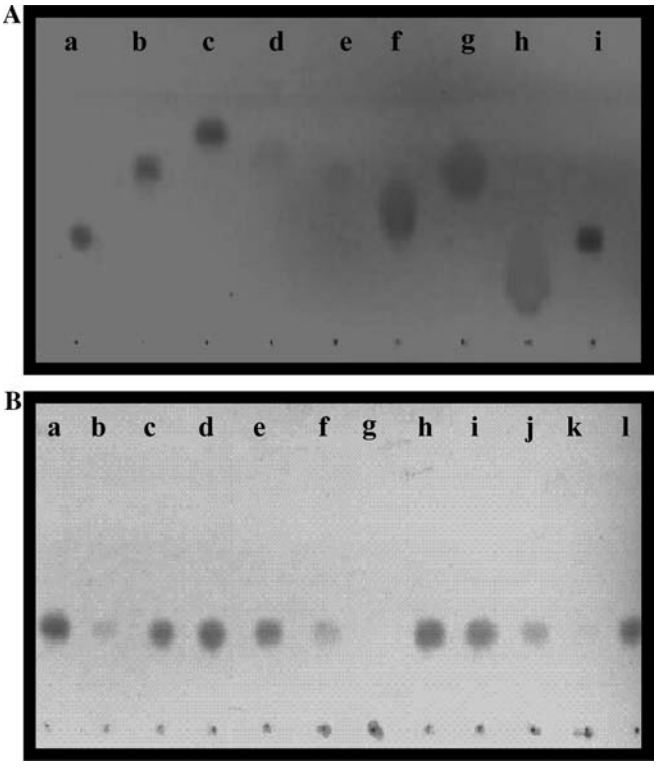

Fig. 3 Thin-layer chromatography of standard sugars (**a**) and dried samples of cell extracts of stationary phase (**b**). **a** Trehalose (*a*), galactose (*b*), glucose (*c*), mannitol (*d*), sorbitol (*e*), maltose (*f*), sucrose (*g*), raffinose (*h*), and trehalose (*i*). **b** Trehalose control (*a*); *S. cerevisiae* La Azteca grown in YPD (*b*); *D. hansenii* (Y7426) grown in different conditions (*c–k*)—C (*c*), Na06 (*d*), Na1 (*e*), Na2 (*f*), Na3 (*g*), K06 (*h*), K1 (*I*), K2 (*j*), K3 (*k*), and trehalose control (*l*)

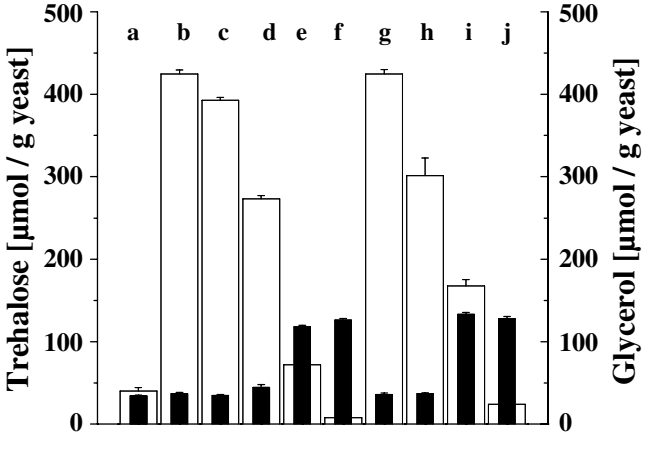

Fig. 4 Trehalose (*empty bars*) and glycerol (*filled bars*) content of *S. cerevisiae* La Azteca cultivated without salt (*a*) and *D. hansenii* Y7426 cultivated in C (*b*), Na06 (*c*), Na1 (*d*), Na2 (*e*), Na3 (*f*), K06 (*g*), K1 (*h*), K2 (*I*), and K3 (*j*). All extracts were obtained at the stationary phase without starvation. Trehalose and glycerol were measured as described under "Materials and methods." Results are the means ± SEM ($n = 3$)

The possible function of trehalose in the metabolism in *D. hansenii* strain Y7426 was analyzed by determining the possible protective effect of this carbohydrate on the enzymatic activity of alcohol dehydrogenase from cells cultivated under different saline-stress conditions. It was found that for this enzyme and under our experimental conditions, glycerol and trehalose did not protect against inactivation produced by the addition of different concentrations of salt in vitro (data no shown).

The possible participation of trehalose as a reserve carbohydrate in *D. hansenii* and *S. cerevisiae* strains was studied. As Table 4 shows, the trehalose content of cells starved during 14 h markedly decreased under all conditions tested, except for those that were already very low at the highest salt concentrations.

The viability of the *D. hansenii* Y7426 cells (Table 5) grown under the different salt concentrations was studied. Moderate saline stress (0.6 and 1.0 M of salt) showed 100% viability. Higher salt concentrations of 2.0 and 3.0 M decreased viability, and the negative effect of NaCl was greater than that of KCl.

It has been reported that trehalose plays an important role in thermal stress in bacteria and yeasts (João et al. 1997; Argüelles 2000; Rontein et al. 2002). The effect of temperature was explored on the production of trehalose in conditions of thermal stress of *D. hansenii* Y7426 for 30 min (Table 6). In general, the production of the disaccharide decreased as the temperature of incubation increased; it is interesting that at low temperature (8°C), an increase of 100–150 µM was observed under all conditions, compared to the usual temperature (30°C). When the cells were incubated at 40 or 50°C, those grown in 1.0 M of NaCl or 0.6 and 1.0 M of KCl showed an increase of 30–100 µM, compared to the control cells (30°C) (see Table 6).

Discussion

Several groups have confirmed that *Debaryomyces hansenii* grows well in the presence of moderate salt concentrations (Neves et al. 1997; Prista et al. 1997; Thomé-Ortíz et al. 1998); however, different *D. hansenii* strains have been used. In this study, we found that under most growth conditions, the presence of moderate salt concentrations favors a better growth. *D. hansenii* Y7426 and CBS1793 strains grew better than the *D. hansenii* J26 strain, and of course, than the *Saccharomyces cerevisiae* strains, which are not salt-tolerant. The results suggest that regarding different strains of *D. hansenii*, this different behavior towards salt, as well as other parameters should be kept in mind.

All strains tested showed the high capacity of *D. hansenii* to accumulate high Na^+ concentrations in comparison to *S. cerevisiae*; however, it is important to note that the capacity of *D. hansenii* Y7426 strain to accumulate this cation was almost twice that, as compared with the other two *D. hansenii* strains used. It should be noted, however, that this same strain, which accumulates more Na^+, also showed the lowest maximum growth (Fig. 1a–c). Norkrans and Kylin (1969) explored the capacity of *D. hansenii* to tolerate high

Table 4 Effect of NaCl and KCl on the trehalose content of *D. hansenii* and *S. cerevisiae* cells starved overnight after growing to the stationary phase

	D. hansenii[a]			*S. cerevisiae*[a]	
	Y7426	J26	CBS1793	La Azteca	ADY
Trehalose (μM)					
C	120.8 ± 1.0	398.9 ± 7.7	378.1 ± 2.7	22.3 ± 0.2	42.1 ± 0.6
Na06	140.6 ± 1.3	343.2 ± 6.7	201.6 ± 5.5	58.7 ± 0.9	137.4 ± 3.1
Na1	74.2 ± 0.7	291.1 ± 3.8	155.6 ± 1.3	2.8 ± 0.3	7.0 ± 0.5
K06	222.3 ± 7.7	337.2 ± 4.2	252.7 ± 9.0	38.2 ± 1.2	63.2 ± 0.7
K1	216.2 ± 5.1	257.5 ± 1.0	140.8 ± 1.1	7.8 ± 0.3	63.7 ± 1.0

[a]After growth, the cells were starved for 14 h and disrupted by boiling the cell suspensions at 92°C in a water bath for 15 min. After centrifugation the supernatant was frozen until analysis. Using adequate dilutions of the supernatants, trehalose was measured as described under "Materials and methods"

Table 5 Viability of *D. hansenii* strain Y7426 grown with different salt concentrations

	Viability[a] (%)
YPD	100
0.6 M NaCl	100
1.0 M NaCl	100
2.0 M NaCl	15
3.0 M NaCl	5
0.6 M KCl	100
1.0 M KCl	100
2.0 M KCl	57
3.0 M KCl	5

[a]After growth, a known number of cells were plated on solid YPD medium, and viability was determined by colony counting. Data are representative of six experiments. Details are described under "Materials and methods"

concentrations of NaCl and found that in its presence, it accumulates high internal concentrations of Na^+. However, when placed in the presence of K^+ or Rb^+, or even water, it rapidly extrudes Na^+ to concentrate K^+ inside, if present (Norkrans and Kylin 1969). Since the net accumulation balance is due to the balance between cation uptake and extrusion (González-Hernández et al. 2004), the differences found with the different species studied may be due to a better Na^+ extrusion and better uptake capacity of K^+ in *D. hansenii*.

Since strain Y7426 showed the highest Na^+ accumulation, it was used in most of the following experiments.

The results show the high capacity of *D. hansenii* to produce trehalose, as compared to *S. cerevisiae*, both under basal and moderate salt stress. Our results suggest a possible role of trehalose in the metabolism of *D. hansenii* as a carbohydrate reserve. The main role of trehalose in fungi has been considered as a storage compound (Thevelein 1984); apparently, in this yeast, it has a similar role. The pure product isolated from the extracts was in fact, trehalose, as demonstrated by comparing the ^{13}C-NMR spectra from the material extracted, and that of the pure sugar, as well as the chromatographic mobility by TLC. Results from both TLC and trehalase assay showed too that concentrations of trehalose diminished under saline stress in *D. hansenii* Y7426 strain.

The protective effects of trehalose are linked to the stabilization of membranes and the preservation of enzyme activity (Crowe et al. 1984; Anchordoguy et al. 1987). The viability and thermal stability of sensitive strains to dehydration can be improved by the addition of certain amounts of disaccharides (trehalose, maltose) to the suspending media in which the cells are to be freeze-dried. Trehalose, like other polyols, could act by replacing water molecules involved in the maintenance of the tertiary structure of proteins through multiple

Table 6 Effect of incubation for 30 min at different temperatures on trehalose content of *D. hansenii* strain Y7426 cells grown to the stationary phase without starvation

	D. hansenii Y7426 (μM)[a]			
	8°C	30°C	40°C	50°C
C	512.5 ± 2.4	424.5 ± 4.9	362.6 ± 14.1	329.8 ± 6.3
Na06	512.3 ± 2.6	392.7 ± 3.7	348.4 ± 4.6	327.4 ± 4.8
Na1	424.6 ± 2.1	273.1 ± 4.0	302.9 ± 11.2	278.5 ± 0.1
K06	512.3 ± 0.5	424.4 ± 5.5	489.9 ± 22.5	422.0 ± 7.4
K1	504.5 ± 1.9	301.3 ± 21.3	403.5 ± 7.8	373.1 ± 11.4

[a]After growth of the cells under the different conditions, they were incubated at different temperatures (8, 30, 40 and 50°C) during 30 min; cellular extracts were obtained by boiling the cells and centrifuging, and trehalose content was determined as described under "Materials and methods." Results are the means of three experiments

external hydrogen bonds (Arakawa and Timasheff 1982; Crowe et al. 1984; Anchordoguy et al. 1987; Stambuk et al. 1993); this may be one of the possible roles of trehalose in *D. hansenii* (Y7426) to explain its high viability under moderate saline stress conditions.

It has been reported that exponential cells improve their stress tolerance towards high salinity by producing and accumulating high intracellular glycerol levels (Adler and Gustafsson 1980; Larsson et al. 1990), whereas stationary-phase cells improve their dehydration resistance upon exposure to saline growth conditions. During this growth phase, they lack glycerol, but accumulate instead high concentrations of arabinitol.

Khroustalyova et al. (2001) found that *D. hansenii* strain J26 cultivated in medium containing 10% (w/v) NaCl, resulted in an additional increase in cellular resistance to dehydration, which was most marked for stationary-phase cells; their results seem to indicate that arabinitol, but not glycerol, might have a protective function in *D. hansenii* J26 strain under dehydration conditions. From our results, we suggest that possibly trehalose was the principal protective disaccharide under dehydration conditions in these experiments; this is also in agreement with the data reported by Wiemken (1995), and Panek (1995).

In several yeasts, defense responses to salt stress are based on osmotic adjustment by osmolyte synthesis and cation transport systems for sodium exclusion. Polyols, especially glycerol, are the major osmolytes produced by yeast (Blomberg and Adler 1992; Brown 1990). To increase osmolarity in the cell under salt stress, glycerol is produced as the major osmolyte by exponentially growing cells in the yeasts *D. hansenii*, *S. cerevisiae*, and *Zygosaccharomyces rouxii* (Gustafsson and Norkrans 1976; Adler et al. 1985; Larsson and Gustafsson 1987; Blomberg and Adler 1989; Yagi 1991). Accumulated glycerol is considered to function as an osmoregulator and an osmoprotector of enzymatic activities under salt stress (Brown and Simpson 1972; Brown 1978). In *D. hansenii* and *Z. rouxii*, the maximal intracellular glycerol concentration increases approximately in proportion to the NaCl concentration of the growth medium (Gustafsson and Norkrans 1976; Yagi 1988). This provides more evidence to support the established opinion about the role of glycerol in cells under salt stress. In our experiments, it was found that *D. hansenii* accumulates more glycerol than trehalose under saline stress (2.0 and 3.0 M of salts). However, under moderate NaCl stress, yeast cells accumulate more trehalose.

The data presented in this comparative analysis might also have biotechnological implications and point to *D. hansenii* as an interesting model cell for further investigations of eukaryotic responses to dehydration stress by industries producing active or instant dry yeasts, searching for new ways to increase the quality of their products. Further studies of the mechanisms behind this extreme resistance to dehydration might provide new avenues for inducing similar resistance in the yeast *S. cerevisiae*. In addition to osmotic stress, high salinity also leads to ion toxicity effects, but specific responses to this form of stress are quite unknown (Serrano 1996).

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