

# Osmotic adaptation of the halophilic fungus *Hortaea werneckii*: role of osmolytes and melanization

Tina Kogej,<sup>1</sup> Marlene Stein,<sup>2</sup> Marc Volkmann,<sup>3†</sup> Anna A. Gorbushina,<sup>3</sup> Erwin A. Galinski<sup>2</sup> and Nina Gunde-Cimerman<sup>1</sup>

## Correspondence

Nina Gunde-Cimerman  
nina.gunde-cimerman@bf.uni-lj.si

<sup>1</sup>University of Ljubljana, Biotechnology Faculty, Department of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia

<sup>2</sup>Institut für Mikrobiologie und Biotechnologie, Rheinische Friedrich-Wilhelms-Universität Bonn, Meckenheimer Allee 168, D-53115 Bonn, Germany

<sup>3</sup>Geomicrobiology, ICBM, Carl von Ossietzky Universität Oldenburg, POB 2503, D-26111 Oldenburg, Germany

This study was intended to determine the osmoadaptation strategy of *Hortaea werneckii*, an extremely salt-tolerant melanized ascomycetous fungus that can grow at 0–5.1 M NaCl. It has been shown previously that glycerol is the major compatible solute in actively growing *H. werneckii*. This study showed that the exponentially growing cells also contained erythritol, arabitol and mannitol at optimal growth salinities, but only glycerol and erythritol at maximal salinities. The latter two were both demonstrated to be major compatible solutes in *H. werneckii*, as their decrease correlated with the severity of hypoosmotic shock. Besides higher amounts of erythritol and lower amounts of glycerol, stationary-phase cells also contained mycosporine-glutaminol-glucoside, which might act as a complementary compatible solute. *H. werneckii* is constitutively melanized under various salinity conditions. Ultrastructural study showed localization of melanin in the outer parts of the cell wall as a distinct layer at optimal salinity (0.86 M NaCl), whereas cell-wall melanization diminished at higher salinities. The role of melanized cell wall in the effective retention of glycerol is already known, and was also demonstrated in *H. werneckii* by lower retention of glycerol in cells with blocked melanization compared to melanized cells. However, these non-melanized cells compensated for the lower amounts of glycerol with higher amounts of erythritol and arabitol. We hypothesize that *H. werneckii* melanization is effective in reducing the permeability of its cell wall to its major compatible solute glycerol, which might be one of the features that helps it tolerate a wider range of salt concentrations than most organisms.

Received 18 June 2007

Revised 28 August 2007

Accepted 7 September 2007

## INTRODUCTION

Water is of vital importance to all organisms. In an aqueous environment with high salt concentration, a consequence of osmosis is loss of internal water (Yancey, 2005). Strategies for cellular osmotic adaptations are conserved from bacteria to man (Klipp *et al.*, 2005). A well-studied model system for research on osmotic adaptation and regulation is the moderately halotolerant baker's yeast *Saccharomyces cerevisiae* (Blomberg, 2000; Hohmann, 2002; Klipp *et al.*, 2005; Mager & Siderius, 2002). A lot of information is available on its osmotic

shock-induced signal transduction, accumulation of osmolytes and control of gene expression (Hohmann, 2002). However, while 3% NaCl represents a concentration that is toxic for *S. cerevisiae*, the same concentration of NaCl is close to the growth optimum for the halotolerant yeast *Debaryomyces hansenii* (Prista *et al.*, 1997) and the halophilic black yeast *Hortaea werneckii* (Gunde-Cimerman *et al.*, 2000). The black yeast *H. werneckii* is a ubiquitous inhabitant of eutrophic hypersaline waters of salterns (Butinar *et al.*, 2005; Cantrell *et al.*, 2006; Gunde-Cimerman *et al.*, 2000) and is one of the most salt-tolerant eukaryotic organisms so far described. It can grow, albeit extremely slowly, in a nearly saturated salt solution (30% or 5.1 M), as well as completely without salt (Gunde-Cimerman *et al.*, 2000). As not many extremely salt-tolerant eukaryotic micro-organisms are known, *H.*

†Present address: Environmental Biochemistry, Carl von Ossietzky Universität Oldenburg, POB 2503, D-26111 Oldenburg, Germany.

Abbreviations: MAA, mycosporine-like amino acid; TEM, transmission electron microscopy.

*werneckii* represents a novel model organism for studying the mechanisms of osmotic adaptation and salt tolerance in eukaryotes (Petrovič *et al.*, 2002). Since the first isolation of *H. werneckii* from hypersaline water in 1997, we have studied various aspects of its adaptation to saline environments (see Plemenitaš & Gunde-Cimerman, 2005 for a review).

Halophilic micro-organisms have developed different strategies to counterbalance changes in osmotic pressure. The strategy of the accumulation of KCl up to molar concentrations in extremely halophilic Archaea requires complete adaptation of the intracellular components, which can function at high intracellular salt concentrations (Oren, 1999). A more widely used strategy, used by most halophilic and halotolerant Bacteria and eukaryotic halophiles (Oren, 1999), involves the cytoplasmic accumulation of 'compatible solutes', low-molecular-mass organic compounds, which provide the cells with osmotic balance and turgor without interference with cellular metabolism (Brown, 1976). At high salinity, halotolerant and halophilic fungi maintain positive turgor pressure mainly by the increased production and accumulation of glycerol. Other compatible solutes include other polyols such as erythritol, inositol, arabitol, xylitol and mannitol (Blomberg & Adler, 1992; Pfyffer *et al.*, 1986), nitrogen-containing compounds such as glycine betaine and free amino acids (Galinski, 1995), sodium (Prista *et al.*, 1997), mycosporines (Kogej *et al.*, 2006b; Oren & Gunde-Cimerman, 2007), and mycosporine-like amino acids (MAAs; Oren, 1997).

Preliminary studies have shown that glycerol is the most important compatible solute in halophilic *H. werneckii*, but also indicated the probable presence of other compatible solute(s) or ions (Petrovič *et al.*, 2002). As we have shown that *H. werneckii* maintains very low intracellular amounts of potassium and sodium even when grown in the presence of high NaCl concentrations (Kogej *et al.*, 2005), osmotic adaptation by using ions may be ruled out.

Based on its constitutive melanization under saline as well as non-saline growth conditions (Kogej *et al.*, 2004), we hypothesized that melanin has a role in the osmoadaptation of *H. werneckii*. In appresoria of the rice pathogenic fungus *Magnaporthe grisea*, melanization of the cell wall is involved in the retention of glycerol and hence is responsible for the generation of very high osmotic pressure, which is important for penetration of plant cells (de Jong *et al.*, 1997).

The objective of this study was to reveal the mechanisms of osmoadaptation of the extremely salt-tolerant black yeast *H. werneckii*. We hypothesized that *H. werneckii* uses a combination of compatible solutes and cell-wall melanization for growth at a range of NaCl concentrations. The types and amounts of intracellular osmolytes were determined at various salinities and after exposure of the salt-adapted *H. werneckii* to hypoosmotic stress. The potential role of melanin in the process of osmotic

adaptation of *H. werneckii* was also studied. The results revealed how this eukaryotic organism is able to tolerate a wide range of salinities using a combination of osmo-adaptation strategies.

## METHODS

**Strain source and preservation.** Halophilic *H. werneckii* (B-736), a black yeast from the ascomycetous order Dothideales, was used in this study. The strain was previously isolated on selective media with 17% (w/v) NaCl from the hypersaline water of a crystallizer pond in the solar saltern Sečovlje, on the eastern coast of the Adriatic Sea (Gunde-Cimerman *et al.*, 2000). It is preserved in the Culture Collection of the National Institute of Chemistry (MZKI), Ljubljana, Slovenia. The strain was kept on solid yeast nitrogen-base (YNB) medium at 4 °C for a few weeks or at -80 °C in 15% (v/v) glycerol for long-term storage.

**Media and growth conditions.** The cultures for biochemical analyses were grown in a defined liquid YNB medium with casamino acids [complete supplement mixture (CSM), both Qbiogene] as described previously (Kogej *et al.*, 2005). Malt-extract medium (MEA) was used for growth of the fungal cultures that were prepared for transmission electron microscopy (TEM). MEA contained 2% (w/v) Bacto malt extract (Difco), 0.1% (w/v) Bacto peptone (Difco), 2% (w/v) glucose, 2% (w/v) agar and NaCl. The salinity of the media was adjusted with various concentrations of NaCl.

**Determination of dry weight.** For dry-weight determination, three 10 ml samples of fungal cultures grown in liquid YNB media amended with various concentrations of NaCl were filtered through Millipore membrane filters (0.45 µm pore-size) and dried at 80 °C to constant weight. The weight was corrected for the salt content of the growth media.

**Osmolytes in salt-adapted cells.** Fungal cultures were grown in triplicate in 100 ml YNB amended with various NaCl concentrations, to the mid-exponential, late-exponential and stationary growth phases, and prepared for HPLC analysis of intracellular osmolytes. Each experiment was performed at least twice. Cultures grown in YNB with 20% (w/v) NaCl were also sampled during the early exponential and late-stationary phases to determine the dynamics of the intracellular osmolyte pool.

**Osmolytes in cells after hypoosmotic shock.** Fungal cultures were grown in 150 ml YNB with either low 5% (w/v) NaCl or high 20% (w/v) NaCl concentrations to the mid-exponential and stationary growth phases, as described above. Each of the four cultures was then divided into three equal volumes and centrifuged at 2655 g (Centrifuge 5810 R, Eppendorf) and 28 °C for 5 min. The supernatant was removed, filtered (0.22 µm pore-size filter) and frozen until analysis. The cells were resuspended in three different dilutions of the growth media [20, 50 and 80% (v/v)], which were prepared from medium of the same salinity by the addition of distilled water. Then the cells were incubated at 180 r.p.m. at 28 °C for 1 h to allow the release of solutes. All experiments were performed in triplicate.

**Osmolytes in melanin-inhibited cultures.** To compare the amount of osmolytes in non-melanized and melanized *H. werneckii*, the cultures were grown in 100 ml YNB with either low [5% (w/v) NaCl] or high [20% (w/v) NaCl] salt content, with and without a melanin-biosynthesis inhibitor. A dihydroxynaphthalene (DHN)-melanin inhibitor, tricyclazole (5-methyl-1,2,4-triazolo(3,4,b)-benzothiazole) (Eli Lilly), was added to the autoclaved YNB as an ethanol solution, so

that the final tricyclazole and ethanol concentrations in YNB were 30  $\mu\text{g ml}^{-1}$  and 1% (v/v), respectively. As a control, the same amount of ethanol, without tricyclazole, was added to YNB. The cultures were grown to the exponential or stationary phase then samples for the analysis of osmolytes were prepared.

**Preparation of samples for HPLC analysis.** Samples of cells grown to the appropriate growth phase [or in the case of hypoosmotic shock, cells after incubation in diluted growth media originally containing 5 or 20% NaCl (for details of dilution see Methods, Osmolytes in cells after hypoosmotic shock)] were filtered through Millipore membrane filters (0.45  $\mu\text{m}$  pore-size), and washed with an isoosmotic (the same NaCl concentration as in the growth medium) ice-cold solution of the same composition as the respective growth medium but without glucose. The cells collected on the filter were washed onto a new filter, and washed again in the same manner. They were scraped off the filter, frozen in liquid nitrogen and freeze-dried. The filtrates were also frozen at  $-20\text{ }^{\circ}\text{C}$  until analysis.

**Extraction of polyols.** Polyols were extracted from 30 mg freeze-dried cells by a modification of the method of Bligh & Dyer (1959) (Galinski & Herzog, 1990). The cells were suspended in 500  $\mu\text{l}$  Bligh & Dyer solution (composed of methanol/chloroform/water, 10/5/4) and vigorously shaken for approximately 30 min (IKA-Vibrax VXR, Janke & Kunkel). Then, 130  $\mu\text{l}$  chloroform and 130  $\mu\text{l}$  demineralized water were added and the suspension was again incubated for 30 min with shaking. The samples were centrifuged at 5510 g (Biofuge A, Heraeus Instruments) for 10 min for phase separation. The upper aqueous phase containing the solutes was transferred to a 1.5 ml tube and frozen at  $-20\text{ }^{\circ}\text{C}$  until analysis. The samples were diluted with an appropriate volume of 80% (v/v) acetonitrile before analysis.

**Detection and characterization of polyols.** HPLC analysis was performed with the Spectra system (Thermo Separation Products) equipped with a P100 pump, UV 1000 UV detector and Shodex RI-71 (Showa Denko) RI detector. HPLC separations were performed with a Grom-Sil Amino-IPR column (3  $\mu\text{m}$ , 125  $\times$  4 mm, LiChrocart system, Grom) with a LiChrospher 100 NH<sub>2</sub> guard column (Merck) at ambient temperature. Acetonitrile (Merck; 80%, v/v) in water (HPLC quality) was used as a mobile phase at a flow rate of 1 ml  $\text{min}^{-1}$ . Then, 20  $\mu\text{l}$  sample was injected using a Rheodyne injector number 7125 (Rheodyne). Chromatogram analysis was performed with the chromatography software ChromQuest version 2.51 (Thermo Quest).

NMR measurements were performed on a Bruker Avance 300DPX unit using D<sub>2</sub>O as a lock signal and trimethylsilylpropionic acid sodium salt (TMSP) as the internal standard. Cell extracts from  $\sim$ 0.5 g dried cells were freeze-dried and resuspended in 1 ml D<sub>2</sub>O.

**Extraction of mycosporines.** The freeze-dried biomass was pulverized under liquid nitrogen using a pestle and mortar. Solid-liquid extraction procedures as described by Volkmann & Gorbushina (2006) were used to extract the UV-absorbing compounds from the samples.

**Detection and characterization of mycosporines.** Mycosporines and MAAs were characterized by a Waters Alliance 2795 HPLC System, equipped with a diode-array detector. A Nucleosil 100RP-18 column (5  $\mu\text{m}$ , 4.6  $\times$  250 mm, Macherey Nagel), equipped with a guard pre-column containing the same material, was used for separation. Then, 20  $\mu\text{l}$  of the sample was injected onto the column using an autosampler system. The solution primarily used for the extraction [0.2% aqueous acetic acid + 0.5% methanol (v/v)] was used as a mobile phase at a flow rate of 0.7 ml  $\text{min}^{-1}$ . The diode-array detector was continuously scanning from  $\lambda=250$  to  $\lambda=400$  nm. The UV-absorbing mycosporines were identified by their

UV-absorption maxima and retention times (Volkmann & Gorbushina, 2006). The fraction containing mycosporine-glutaminol-glucoside was collected and measured spectrophotometrically at 310 nm. The amount of mycosporine-glutaminol-glucoside was quantified as in Libkind *et al.* (2004) using the molar absorption coefficient (25 000  $\text{M}^{-1} \text{cm}^{-1}$ ) from Bouillant *et al.* (1981).

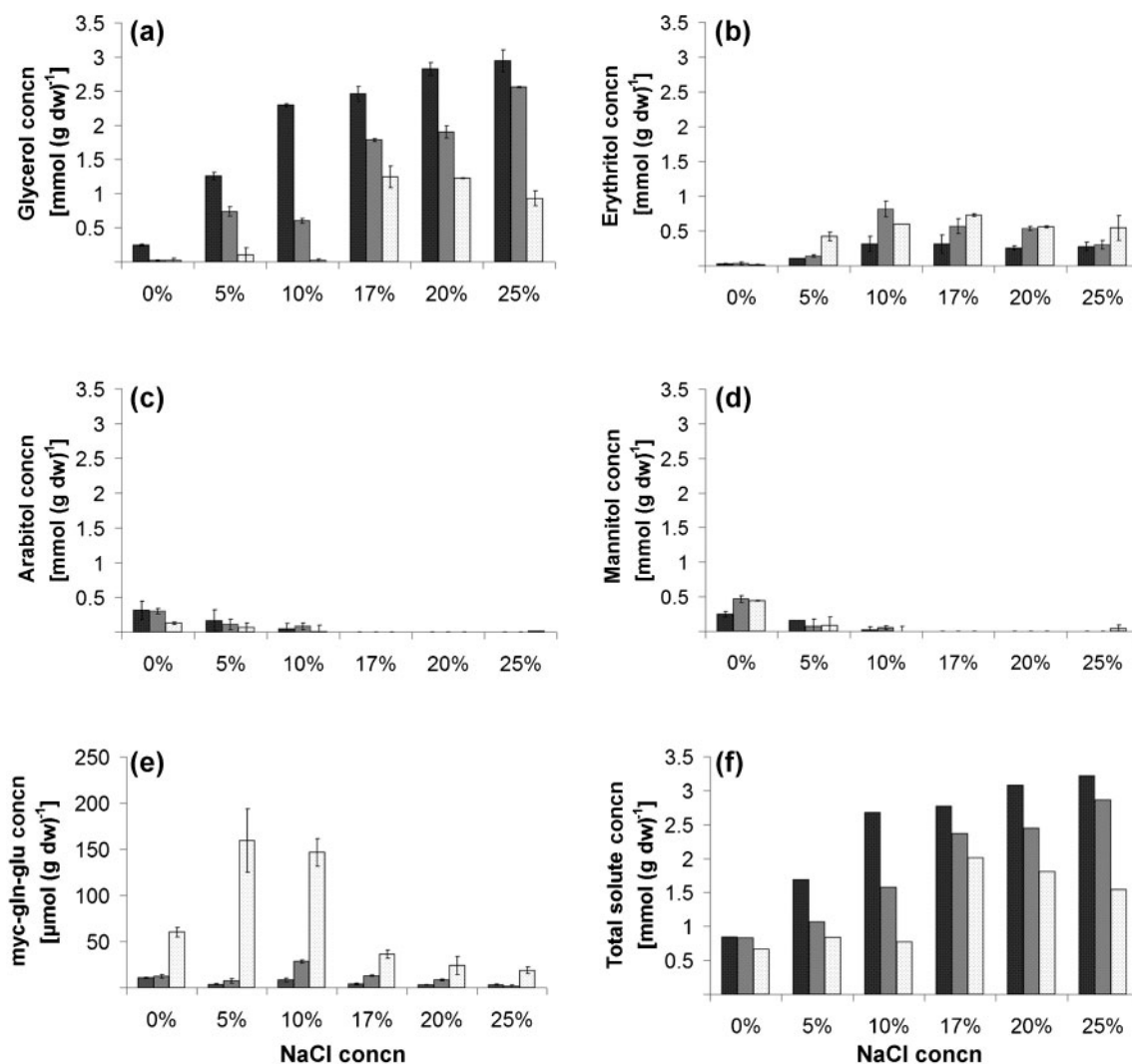
**Ultrastructure.** *H. werneckii* was grown and prepared for TEM, as described previously for *Trimmatostroma salinum* (Kogej *et al.*, 2006a). *H. werneckii* B-736 was grown on solid MEA amended with either no NaCl or 5, 10 or 20% (w/v) NaCl. Tricyclazole was added to autoclaved MEA as an ethanol solution, so that the final concentrations of tricyclazole and ethanol were 30  $\mu\text{g ml}^{-1}$  and 1% (v/v), respectively. Preculturing on the same MEA media as used in the experiment was used to adapt the fungi to the media and growth conditions. The inocula from pre-cultures were prepared as cell suspensions in sterile water of the same salinity and drop-inoculated onto a piece of cellophane overlaid on the surface of solid MEA media. The plates were incubated at 22  $^{\circ}\text{C}$  for 10 days in the dark (Kogej *et al.*, 2006a). Ten-day-old colonies were fixed in 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 h at room temperature, and post-fixed in 1% OsO<sub>4</sub> for 1 h at 4  $^{\circ}\text{C}$ . Embedding in 2% agarose was used after fixation to allow the manipulation of cells. Dehydration was done in a series of graded ethanol solutions: 30% (v/v) (15 min), 50% (2  $\times$  15 min), 70% (2  $\times$  15 min), 90% (2  $\times$  15 min), and absolute ethanol (2  $\times$  15 min, 1  $\times$  30 min). Spurr's resin was used for embedding. Sections were cut with an ultramicrotome (Ultracut, Reichert) and contrasted with uranyl acetate and lead citrate. The sections were examined with a Philips CM 100 transmission electron microscope (80 kV; digital camera Gatan Bioscan Camera 792) (Kogej *et al.*, 2006a).

**Statistical analyses.** The differences among the mean amounts of intracellular osmolytes after hypoosmotic shock determined by HPLC analysis were compared by one-way ANOVA ( $n=3$ ). The total polyol amounts were checked for correlation with dilution of the media. The differences between the mean amounts of intracellular osmolytes in non-melanized and melanized *H. werneckii* were first compared by one-way ANOVA ( $n=3$ ), and then by Tukey's HSD test using Excel and XLSTAT (Addinsoft). Tukey's HSD test was used to test whether the mean amounts of solutes of non-melanized cells differed significantly from the mean amounts of solutes of both melanized controls (melanized unamended cells and melanized ethanol-amended cells).

## RESULTS

### *H. werneckii* accumulates a mixture of organic compounds when grown in saline media

We analysed the extracts of *H. werneckii* cells adapted to growth at various constant NaCl concentrations to detect substances with a possible osmotic role. The cell extracts were screened by two different HPLC methods. The first was optimized to detect uncharged compatible solutes including polyols, and the second detected mycosporines and MAAs. Polyols detected by HPLC were glycerol, erythritol, arabitol and mannitol. These results were also confirmed by NMR. The amounts of these polyols varied with the salinity of the growth medium and with the growth phase of the fungal culture (see Fig. 1). In all growth phases, there was a significant correlation between the intracellular amounts of glycerol and the salinity of the growth medium (exponential and late-exponential phases,



**Fig. 1.** Intracellular amounts of glycerol (a), erythritol (b), arabitol (c), mannitol (d), mycosporine-glutaminol-glucoside (myc-gln-glu; e), and the total amount of polyols and mycosporine-glutaminol-glucoside (f) in salt-adapted cells of *H. werneckii* at various growth phases. *H. werneckii* was grown in YNB of various salinities, and sampled during the mid-exponential (dark-grey bars), late-exponential (medium-grey bars) and stationary growth (light-grey bars) phases; samples were prepared as described in Methods, and analysed by HPLC. Data shown are average and range ( $n=2$ ). dw, Dry weight.

$P=0.01$ ; stationary phase,  $P=0.05$ ). The amount of erythritol was not correlated with salinity. It increased between 0 and 10% (w/v) NaCl and remained at a similar value at higher salinities. Additionally, low amounts of arabitol and mannitol were detected in *H. werneckii* grown at up to 10% NaCl, but their amounts decreased as the salinity increased. At salinities higher than 10% NaCl, only trace amounts of both arabitol and mannitol were detected. The total amount of polyols was correlated with increasing salinity mostly due to increases in glycerol in all growth phases. The combined amounts of erythritol, arabitol and mannitol correlated with salinity only in the exponential growth phase (data not shown; sum of values in Fig. 1b–d).

In addition to polyols, *H. werneckii* also accumulated different amounts of two mycosporines at various salinities. Mycosporine-glutaminol-glucoside accumulated in the salt-adapted stationary-phase cells of *H. werneckii* (see Fig. 1e). Its amount increased steeply from 0 to 5% (w/v) NaCl, and decreased at higher NaCl concentrations, whereas mycosporine-glutamicol-glucoside appeared only in very low amounts [up to  $0.2 \mu\text{mol (g dry weight)}^{-1}$ ] in the stationary-phase cells at all salinities (data not shown). Interestingly, the amount of mycosporine-glutaminol-glucoside in the stationary-phase cells was strongly correlated ( $P=0.01$ ) with *H. werneckii* growth rates at the same salinities (Kogej *et al.*, 2005).

We followed the growth-phase dependence of polyol and mycosporine accumulation in *H. werneckii* grown at 20% (w/v) NaCl (Fig. 2). Glycerol accumulated predominantly in the exponential growth phase and diminished steeply in the stationary phase. On the other hand, the amount of erythritol increased gradually during the exponential growth phase and reached its highest level in the stationary phase. The amounts of mycosporine-glutaminol-glucoside and mycosporine-glutamicol-glucoside also increased steeply as the culture entered the stationary phase.

### Only glycerol and erythritol are lost from the cells during hypoosmotic shock

We performed moderate hypoosmotic shock on salt-adapted *H. werneckii* grown at optimal salinity [5% (w/v) NaCl] and drastic hypoosmotic shock on cells grown at high salinity [20% (w/v) NaCl], and measured the intracellular amounts of polyols and mycosporines after the shock, to test their osmotic role. The mid-exponential and stationary-phase cells of *H. werneckii* were incubated in media diluted by 20, 50 and 80% (v/v) for 1 h.

In cells grown at optimal salinity (5% NaCl) to the mid-exponential growth phase, moderate hypoosmotic shock caused a decrease in the intracellular amount of glycerol and a slight decrease of arabitol that was correlated with dilution. However, the amounts of erythritol and mannitol did not change significantly (Fig. 3a). On the other hand, drastic hypoosmotic shock caused a significant decrease in the intracellular amount of glycerol and a slight decrease in erythritol in cells grown at high salinity (Fig. 3c). The amounts of mycosporine-glutaminol-glucoside and mycosporine-glutamicol-glucoside in mid-exponential cells of *H. werneckii* were unaffected by hypoosmotic shock (data not shown).

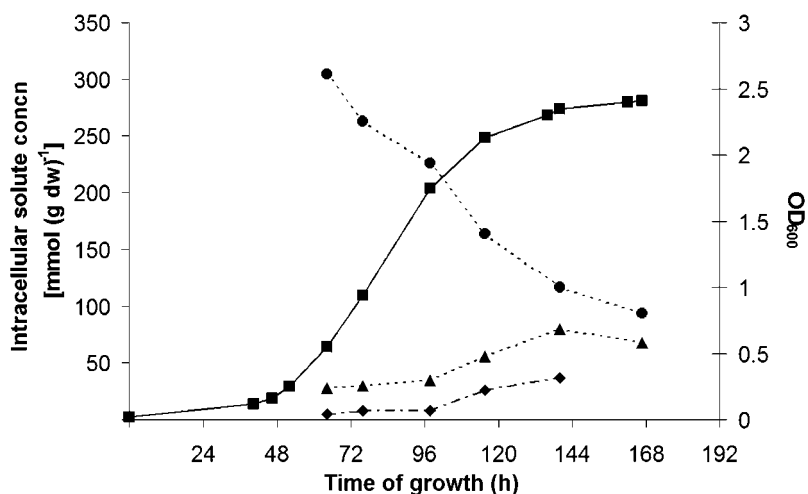
When cells grown at 5% NaCl to the stationary growth phase were exposed to moderate hypoosmotic shock, none of the four polyols changed significantly in level, and

consequently the total amount of polyols remained almost constant (Fig. 3b). Similarly, there was no change in the amount of mycosporine-glutaminol-glucoside or mycosporine-glutamicol-glucoside (data not shown). On the other hand, the strong decrease in both glycerol and erythritol in the drastically hypoosmotically shocked stationary-phase cells of *H. werneckii* grown at 20% NaCl strongly correlated ( $P=0.05$ ) with the dilution of the medium (Fig. 3d). The amount of mycosporine-glutaminol-glucoside increased significantly compared to the non-shocked cells, but the total amounts were low [up to  $0.008 \mu\text{mol} (\text{g dry weight})^{-1}$ ]. In conclusion, moderate hypoosmotic shock in mid-exponential phase cells of *H. werneckii* was most efficiently prevented by lowering the amount of the main compatible solute, glycerol, whereas in stationary-phase cells there was no significant change in polyols. On the other hand, drastic hypoosmotic shock in either mid-exponential or stationary-phase cells grown at 20% NaCl caused loss of glycerol and, to a lesser extent, of erythritol also (mainly in stationary-phase cells).

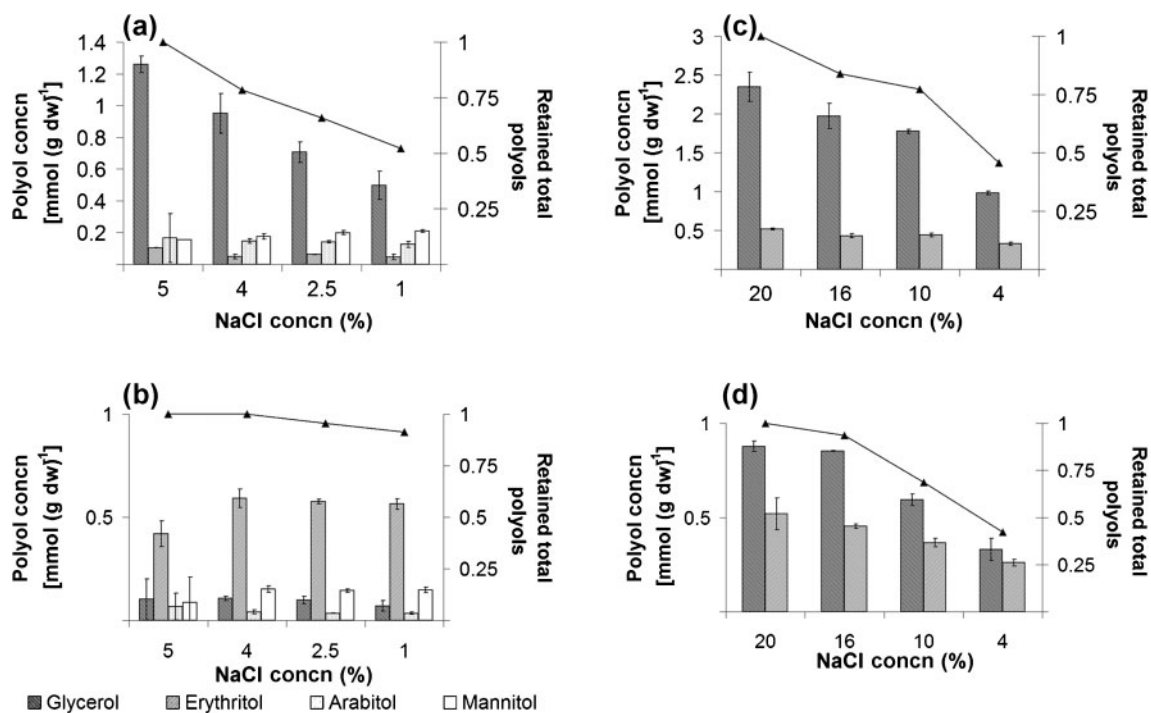
### Salinity affects cell-wall melanization in *H. werneckii*

The melanization of *H. werneckii* was studied at various salinities by TEM. The ultrastructure of melanized cells was compared to that of cells grown in the presence of the melanization inhibitor tricyclazole, a specific inhibitor of trihydroxynaphthalene reductase (Andersson *et al.*, 1996). In melanized cells, melanin was observed as electron-dense granules (dark) in or on the electron-translucent (light-coloured) cell walls, whereas the cells with blocked melanin biosynthesis either had no electron-dense granules or these were smaller and lighter in colour (compare left and right columns in Fig. 4).

In *H. werneckii* cells grown without NaCl, melanin granules were deposited in the outer layer of the cell wall, forming a thin coalescing layer of melanin with separate larger granules in the cell wall. When grown at optimal salinity



**Fig. 2.** Growth phase-dependent accumulation of glycerol (●), erythritol (▲) and mycosporines (◆) in the cells of *H. werneckii* grown at 20% (w/v) NaCl. The cultures were grown in liquid YNB medium with 20% (w/v) NaCl at  $27 \pm 1^\circ\text{C}$  on a rotary shaker and harvested at various growth phases. Growth was monitored by OD<sub>600</sub> (■). The cell samples were extracted and measured by HPLC, as described in Methods. Data are taken from a single experiment.



**Fig. 3.** Effect of hypoosmotic shock on the intracellular amounts of glycerol, erythritol, arabitol and mannitol, and their total amounts ( $\blacktriangle$ ) in mid-exponential phase (a, c) and stationary-phase (b, d) cells of *H. werneckii*. *H. werneckii* was grown in YNB with 5 or 20% (w/v) NaCl, exposed to diluted media of lower salinity for 1 h, prepared as described in Methods, and analysed by HPLC. Data shown are means  $\pm$  SD ( $n=3$ ). (a) Mid-exponential phase, 5% NaCl; (b) stationary phase, 5% NaCl; (c) mid-exponential phase, 20% NaCl; (d) stationary phase, 20% NaCl. dw, Dry weight.

(5% NaCl), *H. werneckii* had a dense shield-like layer of electron-dense coalescing melanin granules on the outer side of the cell wall, also extending into the outer layer of the cell wall (Fig. 4a, c). At 10% NaCl, the electron-dense granules were deposited mostly on the cell wall, they were larger and scarce, and they did not form a continuous layer. On the other hand, in cells grown at 20% NaCl, melanin granules were smaller and deposited in the outer layer of the cell wall (Fig. 4e, g). On tricyclazole-amended media, no melanin granules were visible in the cell wall, but separate smaller and lighter electron-dense granules were present on the outside of the cells (Fig. 4, right column). In conclusion, *H. werneckii* is most melanized at low salinities close to the growth optimum, whereas melanization is reduced at higher salinities.

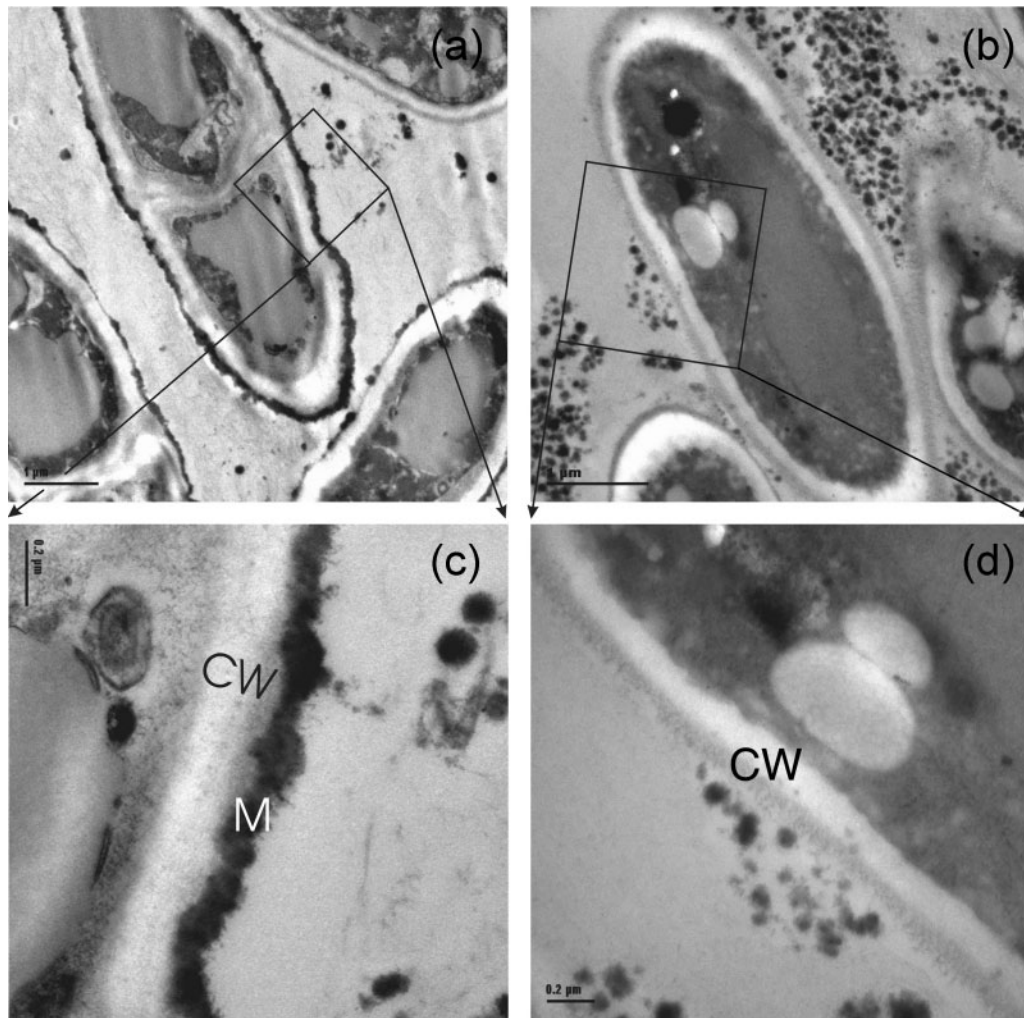
### Non-melanized *H. werneckii* cells retain less glycerol than melanized cells

We assessed the effect of blocked cell-wall melanization on polyol and mycosporine amounts in mid-exponential and stationary-phase *H. werneckii* cells grown with 5 and 20% NaCl.

In mid-exponential phase *H. werneckii* cells grown at 5% NaCl, non-melanized cells contained a significantly lower

amount of glycerol ( $P=0.01$ ) than melanized control cells, while the amounts of erythritol, arabitol and mannitol were higher than in melanized cells. The increased levels of erythritol and arabitol apparently replaced the diminished glycerol content (Fig. 5a). As a result, the total amount of polyols in melanin-inhibited and melanized cells did not change. The comparison of mean amounts of polyols by Tukey's HSD test showed that the amounts of glycerol, erythritol and arabitol in melanin-inhibited cells differed significantly ( $P=0.01$ ) from those in melanized cells. On the other hand, the test showed no significant differences between the amounts of mannitol in melanized and non-melanized cells ( $P=0.05$ ). Interestingly, in mid-exponential *H. werneckii* cells grown at 20% NaCl, tricyclazole inhibition resulted in a significant increase in erythritol content in non-melanized cells compared to the melanized controls, while there was no statistically significant change in glycerol content (Fig. 5c). The amounts of mycosporines were low and were unaffected by tricyclazole inhibition (data not shown).

Stationary-phase cells grown at optimal salinity (5% NaCl) contained almost no glycerol, but mostly erythritol, arabitol and mannitol (Fig. 5b), and also mycosporine-glutaminol-glucoside (Fig. 6a). Stationary-phase cells with blocked melanin synthesis grown at optimal salinity (5%

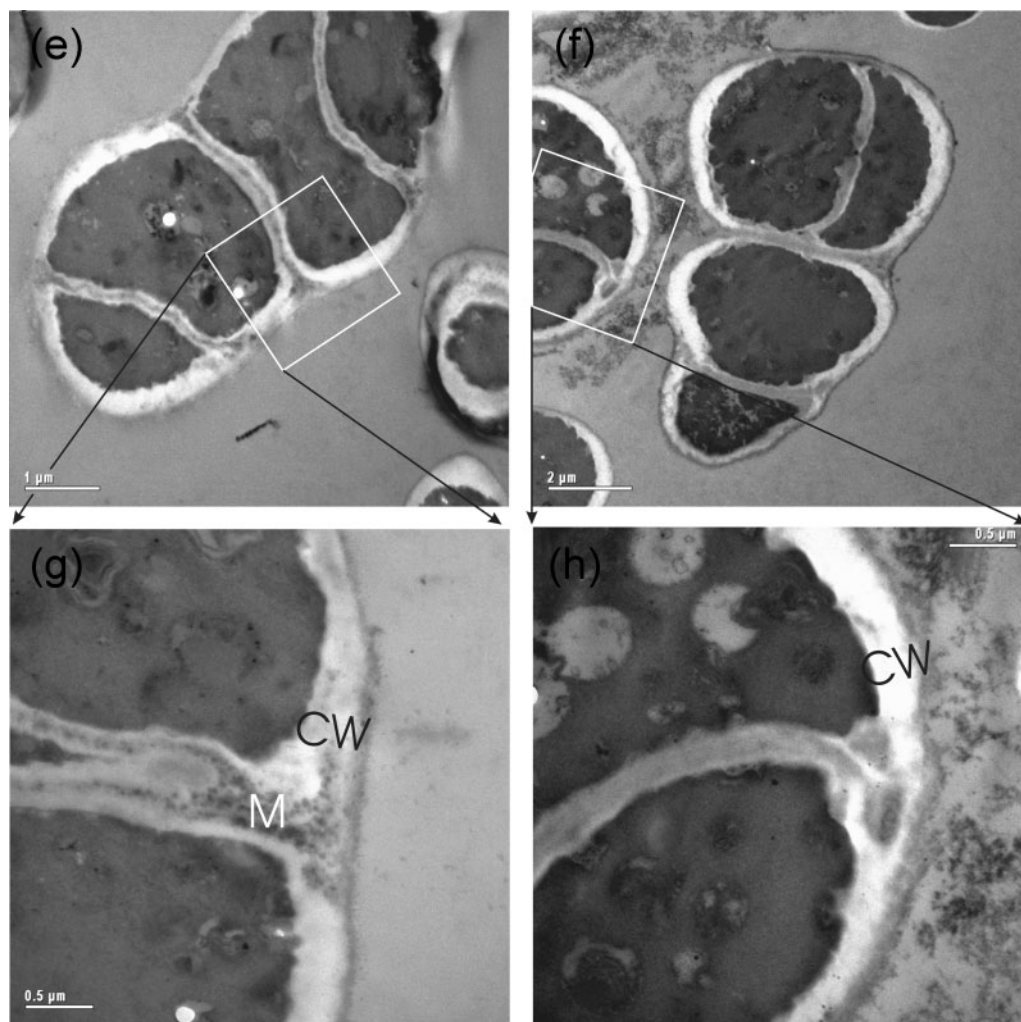


NaCl) contained significantly less erythritol ( $P=0.01$ ) and significantly more arabitol ( $P=0.01$ ) than melanized cells, while the amount of mannitol was significantly higher ( $P=0.01$ ) in both types of control cells (melanized ethanol-amended and non-melanized tricyclazole-amended) than in melanized cells grown in unamended medium. The amount of mycosporine-glutaminol-glucoside was also significantly lower in non-melanized cells than in melanized cells (Fig. 6a). Melanin inhibition of stationary-phase cells grown at 20% NaCl resulted in significantly lower amounts of glycerol ( $P=0.05$ ), while the amounts of erythritol were increased compared to both controls (Fig. 5d). The tricyclazole-inhibited cells contained no mycosporines, while the control cells contained some mycosporine-glutaminol-glucoside (Fig. 6b).

## DISCUSSION

Since 2000, *H. werneckii* has become known as an extremely salt-tolerant model yeast organism. Although its ecology and physiology are well known, the study of its osmoadaptation strategy has been limited to intracellular

glycerol (Petrovič *et al.*, 2002) and ion measurements (Kogej *et al.*, 2005). It has been determined that the amounts of ions remain low at all salinities (Kogej *et al.*, 2005), while the amount of glycerol correlates with increasing salinity in the medium (Petrovič *et al.*, 2002). The main goal of this study was to unravel the complete osmoadaptation strategy of *H. werneckii*. Our results confirm that glycerol is its main compatible solute, since its intracellular amount was the highest of all solutes and it increased with salinity. It accumulated maximally up to  $2.94 \text{ mmol (g dry weight)}^{-1}$  during exponential growth at 25% NaCl. However, *H. werneckii* also compensated for the external osmotic pressure caused by increased NaCl concentrations by accumulating various additional substances (erythritol, arabitol, mannitol and mycosporines). The composition of this mixture depended not only on the external salinity, but also on the age of the cells. Similar observations have been made for some halotolerant/halophilic eubacteria (Galinski & Trüper, 1994), which can change the composition of the solute pool according to growth phase and growth medium. In some halotolerant yeast species, the amount and type of polyol accumulation are also related to the growth phase (Nobre & da Costa,

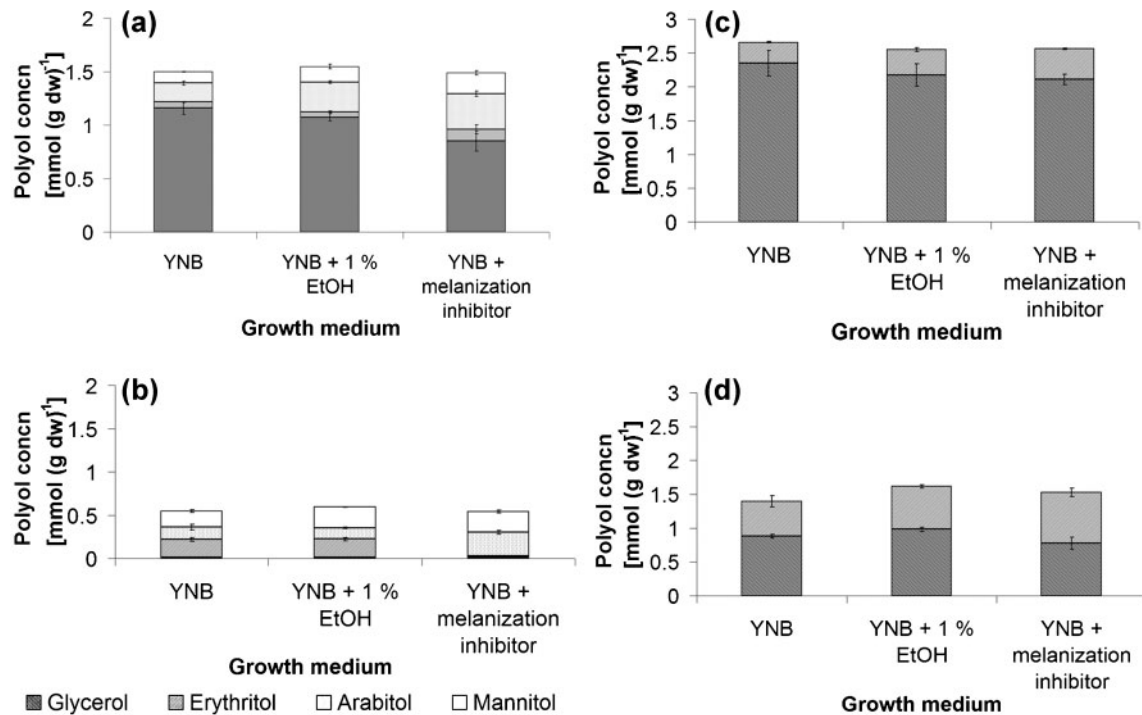


**Fig. 4.** (a–d) TEM micrographs of thin sections of *H. werneckii* cells grown at optimal salinity (MEA medium with 5%, w/v, NaCl). (a, c) Normally melanized cells with visible cell-wall melanization; (b, d) non-melanized cells grown with tricyclazole. (e–h) TEM micrographs of thin sections of *H. werneckii* cells grown at high salinity (MEA medium with 20%, w/v, NaCl). (e, g) Normally melanized cells with separate melanin granules in the cell wall; (f, h) non-melanized cells grown with tricyclazole. *H. werneckii* was grown and prepared for TEM as described in Methods. CW, cell wall; M, melanin.

1985) and the carbon sources used for growth (Van Eck *et al.*, 1989).

Glycerol is known to be the primary osmolyte in many eukaryotic cells, such as in the halophilic green algae *Dunaliella* sp. (Ben-Amotz & Avron, 1973) and *Asteromonas gracilis* (Oren, 2002), and in fungi and yeasts, such as *Aspergillus niger* (Witteveen & Visser, 1995), *S. cerevisiae* (Blomberg & Adler, 1992), *Debaryomyces hansenii* (Adler *et al.*, 1985), *Zygosaccharomyces rouxii* (Kayingo *et al.*, 2001) and *Yarrowia lipolytica* (Andreishcheva *et al.*, 1999). Besides glycerol, other polyols such as erythritol, arabitol and mannitol are also recognized to be compatible solutes in fungi. The strategy of accumulating a mixture of

polyols is also common in many fungi (Davis *et al.*, 2000) and is not unique to *H. werneckii*. For example, *Candida sake* accumulates equimolar amounts of glycerol and arabitol (Abadias *et al.*, 2000). Erythritol is produced by osmophilic yeasts such as *Pichia*, *Candida*, *Torulopsis*, *Trigonopsis*, *Moniliella* and *Aureobasidium* sp. (Kim *et al.*, 1997). All four polyols have been detected in some *Aspergillus* species (Beever & Laracy, 1986; Nesci *et al.*, 2004) and in *C. sake* (Abadias *et al.*, 2000). In addition to their use as compatible solutes, polyols can also serve as carbon storage compounds, they may help in balancing the cellular redox potential (Diano *et al.*, 2006), and they can act as scavengers of reactive oxygen species (Voegelé *et al.*, 2005).



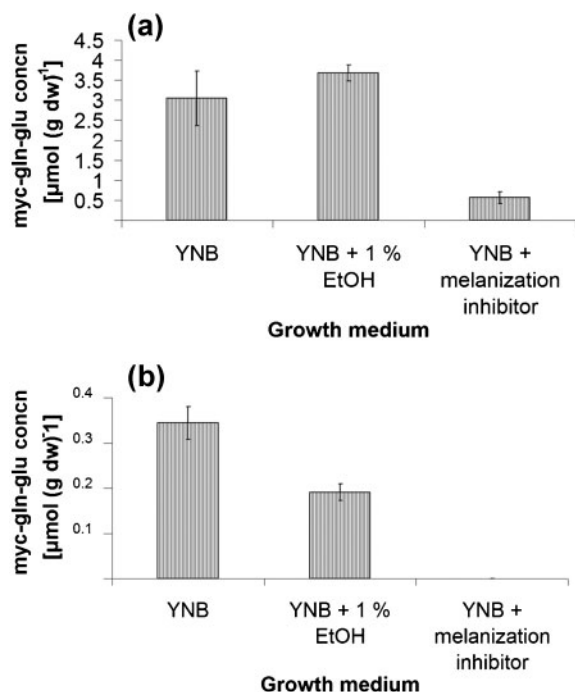
**Fig. 5.** Intracellular polyol amounts in mid-exponential and stationary-phase cells of *H. werneckii* with inhibited melanin biosynthesis. *H. werneckii* was grown in YNB with 5% (a, b) and 20% (w/v) NaCl (c, d) in unamended YNB, in YNB with 1% (v/v) ethanol (EtOH), and in YNB with 1% (v/v) ethanol and 30  $\mu\text{g ml}^{-1}$  tricyclazole (YNB + melanization inhibitor) to the mid-exponential (a, c) or stationary growth phase (b, d). Samples were prepared as described in Methods, and analysed by HPLC. Data shown are means  $\pm$  SD ( $n=3$ ). (a) Mid-exponential phase, 5% NaCl; (b) stationary phase, 5% NaCl; (c) mid-exponential phase, 20% NaCl; (d) stationary phase, 20% NaCl. dw, Dry weight.

At optimal growth salinity, *H. werneckii* accumulated a mix of polyols with different lengths of carbon backbone (from three to six atoms) in various ratios. Experimental osmometric data of Davis *et al.* (2000) show that complex patterns of osmolyte accumulation cannot be explained by their effects upon the cytoplasmic osmotic pressure, because millimolar concentrations of the individual polyols, accumulated by *H. werneckii*, generate similar osmotic pressures (Davis *et al.*, 2000). Such mixtures may reduce the toxicity associated with high concentrations of a single osmolyte, and obviate feedback mechanisms that down-regulate metabolic pathways in the presence of a high concentration of the product (Davis *et al.*, 2000). As salinity increased over the optimal range for *H. werneckii*, the production and accumulation of glycerol rose, while the amounts of other polyols remained low. Interestingly, the osmometric data of Davis *et al.* (2000) also show that at high salt concentrations high-molecular-mass polyols generate higher osmotic pressures than lower-molecular-mass polyols at the same concentration. On the other hand, glycerol synthesis is energetically the least expensive option for cells growing at the highest salinities, since glycerol is energetically the cheapest polyol to produce (Oren, 1999).

Accordingly, glycerol is the main compatible solute in actively growing fungal cells in most cases. However, in *H.*

*werneckii*, the amount of glycerol was drastically reduced in the stationary phase. Since glycerol was only partially replaced by higher levels of erythritol, the total amount of polyols decreased in the stationary phase. A similar shift in polyol pools has been observed in halotolerant *D. hansenii*, which accumulates glycerol in the exponential phase (André *et al.*, 1988) and arabitol in stationary-phase cells (Adler & Gustafsson, 1980), whereas *A. niger* replaces glycerol with mannitol and erythritol in older mycelia (Witteveen & Visser, 1995). Some investigated fungi use high-affinity glycerol transporters to maintain high levels of glycerol, despite continuous efflux (Blomberg & Adler, 1992). Assuming that such transporters exist in *H. werneckii*, a possible explanation for glycerol loss and its partial replacement with erythritol in the stationary phase is a lack of energy for the functioning of glycerol-uptake systems due to a gradual depletion of the substrate. However, to our knowledge, no such transporters have been identified in *H. werneckii* to date.

The response of *H. werneckii* to increased salinities is in some way similar to that of other halotolerant yeast species. Both glycerol and erythritol have been documented as compatible osmolytes in yeast, but not mycosporines, which might act as additional compatible solutes in *H. werneckii*. Mycosporines, substances with an aminocyclohexenone unit



**Fig. 6.** Intracellular amounts of mycosporine-glutaminol-glucoside (myc-gln-glu) in stationary-phase cells of *H. werneckii* with inhibited melanin biosynthesis. *H. werneckii* was grown in YNB with 5% (a) and 20% (b) (w/v) NaCl in unamended YNB, in YNB with 1% (v/v) ethanol (EtOH), and in YNB with 1% (v/v) ethanol and  $30 \mu\text{g ml}^{-1}$  tricyclazole (YNB+melanization inhibitor) to stationary phase. Samples were prepared as described in Methods, and analysed by HPLC. Data shown are means  $\pm$  SD ( $n=3$ ). (a) 5% NaCl; (b) 20% NaCl. dw, Dry weight.

bound to an amino acid or amino alcohol group, were initially known as morphogenetic factors in fungal sporulation and as UV-protecting compounds (Bandaranayake, 1998). Recently, it has been hypothesized that in certain micro-organisms, mycosporines or MAAs play a role as complementary compatible solutes (Kogej *et al.*, 2006b; Oren, 1997; Oren & Gunde-Cimerman, 2007). In an earlier study, a higher amount of mycosporine-glutaminol-glucoside was measured in *H. werneckii* and in the cells of other black yeasts from a hypersaline environment (*Phaeotheca triangularis* and *Aureobasidium pullulans*) grown at 10% NaCl than on non-saline growth medium (Kogej *et al.*, 2006b), with a marked increase of mycosporines in the stationary-phase cells of *H. werneckii*. Interestingly, the amounts of mycosporines correlated with growth rates of *H. werneckii*. The highest amount of mycosporine-glutaminol-glucoside [ $158 \mu\text{mol (g dry weight)}^{-1}$ ] was detected in *H. werneckii* cells grown at optimal salinity (5% NaCl) in the stationary phase. Here, mycosporine-glutaminol-glucoside represented 18.9 mol% of the total osmolytes, while there were 12 mol% glycerol and 50.3 mol% erythritol, in comparison. These results indicate that

mycosporine-glutaminol-glucoside can be considered to be a complementary osmolyte in *H. werneckii* in the stationary phase.

Cells slowly adjusting to various salt concentrations, as described above, differed considerably from cells of *H. werneckii* exposed to sudden hypoosmotic shock. The shocked cells responded by lowering glycerol and erythritol, while the amounts of higher polyols and mycosporines remained unchanged. Thus, the hypoosmotic shock confirmed the role of glycerol as the main compatible solute, which was lowered to balance the sudden change in osmotic pressure. It seems that erythritol acts in the same way in the stationary-phase cells. At present, it is not, to our knowledge, known how *H. werneckii* is able to lower the amount of both polyols. In *S. cerevisiae*, an aquaglyceroporin channel Fps1p opens during steady-state growth. The combination of a closed Fps1p channel and upregulated glycerol production has been found to cause a marked increase in steady-state intracellular glycerol (Klipp *et al.*, 2005). Fps1p is also open when *S. cerevisiae* cells are exposed to a sudden lowering of salinity in their environment, and allows for quick glycerol expulsion during a hypoosmotic shock (Luyten *et al.*, 1995).

Glycerol presents a paradox for cells that hyperaccumulate compatible osmolytes. Due to its small molecular mass, it has an unusually high permeability coefficient for passage through the lipid bilayers relative to other uncharged, polar molecules. Thus, passive efflux from the cytoplasm would seem likely to limit glycerol accumulation (Blomberg & Adler, 1992). In the halophilic alga *Dunaliella* sp., the membrane permeability for glycerol is considerably lowered (Brown *et al.*, 1982; Gimmler & Hartung, 1988), which is correlated with its high sterol content (Oren, 1999; Sheffer *et al.*, 1986). In *H. werneckii* and other halophilic and halotolerant melanized fungi, ergosterol as the principal sterol and 23 other types of sterols (Turk *et al.*, 2004) constitute the most distinctive lipid fraction of the cell membranes (Méjanelle *et al.*, 2000). However, in *H. werneckii* the total sterol content remained largely unchanged with increased salinity, and the cells maintained high membrane fluidity over a broad range of salinities. Membrane fluidity was lower than in *S. cerevisiae* and in the halotolerant *A. pullulans* (Turk *et al.*, 2004). Seemingly, there is a contradiction in the facts that (i) *H. werneckii* can grow at very high salinities, which require a high intracellular amount of glycerol, and (ii) at the same time it maintains a very fluid membrane by keeping a low sterol-to-phospholipid ratio, which does not prevent glycerol leakage. However, adaptation at the cell-wall level can minimize glycerol loss from the cells. For example, the melanized appressorial cell wall in the plant-pathogenic fungus *M. grisea* limits the loss of intracellular glycerol by creating a permeability barrier. This adaptation is crucial for generating a very high turgor by glycerol accumulation (Davis *et al.*, 2000; de Jong *et al.*, 1997).

*H. werneckii* also has melanized cell walls. The ultrastructural studies showed that the salinity of the growth

medium affects the melanization patterns of *H. werneckii*. At 0–5% NaCl, the melanin granules form a thin continuous layer in the outer part of the cell wall (Fig. 4a, c) and could thus create a mechanical permeability barrier for glycerol. Melanin granules probably limit cell-wall permeability by reducing the size of pores in the cell wall, as has been shown for *Cryptococcus neoformans*, in which cell-wall melanization reduces the size of pores from 10.6 nm in non-melanized to 4 nm in melanized cell walls (Jacobson & Ikeda, 2005). Indeed, when cell-wall melanization in *H. werneckii* was blocked by tricyclazole, the non-melanized cells accumulated significantly lower amounts of glycerol and significantly higher amounts of higher-molecular-mass polyols (see Fig. 6). This indicates that melanization limits the permeability of the cell wall to glycerol, the smallest of the detected polyols and the major compatible solute in *H. werneckii*. However, in *H. werneckii* grown at 10 and 20% NaCl, the melanin granules are separate and do not form a continuous layer. Here, melanin probably has a less pronounced effect on the permeability, as is shown by the smaller difference in the glycerol content between melanized and non-melanized cells (Fig. 5c, d). Higher permeability to glycerol would also explain the higher extracellular levels of glycerol detected at high salinities in a previous study of *H. werneckii* (Petrović *et al.*, 2002).

Cell-wall melanization is an important mechanism that helps to maintain a high intracellular concentration of glycerol in *H. werneckii* despite its highly fluid membrane. A melanized cell wall might decrease the energetic needs of the cell, as glycerol accumulation from the medium as well as *de novo* synthesis can be reduced due to more effective glycerol retention. At optimal salinities, *H. werneckii* probably maintains a balance between two options: (i) an energetically cheap production of glycerol, which partially leaks out of the cells and therefore needs to be recovered by energy-dependent processes; and (ii) an energetically more costly synthesis of other compatible solutes, which escape less easily from the cells and are therefore retained more efficiently. At higher salinities, the cost of maintaining osmotic balance in the cells is probably much higher because of the higher concentration of glycerol needed for osmoadaptation, but also due to increased leakage of glycerol as *H. werneckii* cell walls are less melanized. Despite the choice of the shortest polyol as osmolyte at high salinities, which is energetically the cheapest to produce, the higher energetic demands of *H. werneckii* are reflected in reduced growth rates and biomass yield at salinities above 17% NaCl (T. Kogej & N. Gunde-Cimerman, unpublished data). Perhaps the higher proportion of polymorphic cells that were observed with increasing salinity (T. Kogej & N. Gunde-Cimerman, unpublished data) is another mechanism for reducing glycerol leakage when melanization is diminished. Similar observations on the salinity-affected cell-wall melanization patterns of the other halophilic black yeast species *T. salinum* (Kogej *et al.*, 2006a) and *P. triangularis* (T. Kogej &

N. Gunde-Cimerman, unpublished data), which grow at similar salinities as *H. werneckii*, confirm our findings. This might indicate that melanization also has a role in the osmoadaptation of these fungi, but further studies are needed to confirm this.

In conclusion, the main compatible solute of *H. werneckii* is glycerol, which is replaced by erythritol and partially by mycosporine-glutaminol-glucoside in stationary-phase cells. At low salinities, *H. werneckii* accumulates a mixture of glycerol, erythritol, arabinol and mannitol, whereas glycerol and erythritol prevail at high salinities. The role of glycerol and erythritol as the most important osmolytes was confirmed by hypoosmotic shock. At optimal growth salinities, the melanized cell walls help in retaining high concentrations of glycerol in the cells of *H. werneckii*, despite the highly fluid membrane. The novelty of osmoadaptation of the halophilic fungus *H. werneckii*, probably contributing to its growth in a wide salinity range, is an effective combination of the accumulation of known compatible solutes (polyols) and of melanized cell walls for improved osmolyte retention. In combination, an array of adaptations at the levels of osmolyte production, membrane structure and cell-wall melanization interact to make *H. werneckii* a very versatile halophile, able to grow in a broader salinity range than most known micro-organisms.

## ACKNOWLEDGEMENTS

This work was supported by the Ministry of Higher Education and Technology of the Republic of Slovenia in the form of a young researcher's grant to T.K. The financial support of the Ministry of Science and Culture of Lower Saxony (Dorothea Erxleben Program) to A.A.G. is deeply appreciated.

## REFERENCES

- Abadias, M., Teixidó, N., Usall, J., Viñas, I. & Magan, N. (2000). Solute stresses affect growth patterns, endogenous water potentials and accumulation of sugars and sugar alcohols in cells of the biocontrol yeast *Candida sake*. *J Appl Microbiol* **89**, 1009–1017.
- Adler, L. & Gustafsson, L. (1980). Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of the yeast *Debaryomyces hansenii*. *Arch Microbiol* **124**, 123–130.
- Adler, L., Blomberg, A. & Nilsson, A. (1985). Glycerol metabolism and osmoregulation in the salt-tolerant yeast *Debaryomyces hansenii*. *J Bacteriol* **162**, 300–306.
- Andersson, A., Jordan, D., Schneider, G. & Lindqvist, Y. (1996). Crystal structure of the ternary complex of 1,3,8-trihydroxynaphthalene reductase from *Magnaporthe grisea* with NADPH and an active-site inhibitor. *Structure* **4**, 1161–1170.
- André, L., Nilsson, A. & Adler, L. (1988). The role of glycerol in osmotolerance of the yeast *Debaryomyces hansenii*. *J Gen Microbiol* **134**, 669–677.
- Andreishcheva, E. N., Isakova, E. P., Sidorov, N. N., Abramova, N. B., Ushakova, N. A., Shaposhnikov, G. L., Soares, M. I. M. & Zvyagil'skaya, R. A. (1999). Adaptation to salt stress in a salt-tolerant strain of the yeast *Yarrowia lipolytica*. *Biochemistry (Moscow)* **64**, 1061–1067.

- Bandaranayake, W. M. (1998).** Mycosporines: are they nature's sunscreens? *Nat Prod Rep* **15**, 159–172.
- Beever, R. E. & Laracy, E. P. (1986).** Osmotic adjustment in the filamentous fungus *Aspergillus nidulans*. *J Bacteriol* **168**, 1358–1365.
- Ben-Amotz, A. & Avron, M. (1973).** The role of glycerol in the osmotic regulation of the halophilic alga *Dunaliella parva*. *Plant Physiol* **51**, 875–878.
- Bligh, E. G. & Dyer, W. J. (1959).** A rapid method of lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.
- 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.
- Blomberg, A. & Adler, L. (1992).** Physiology of osmotolerance in fungi. *Adv Microb Physiol* **33**, 145–212.
- Bouillant, M.-L., Pittet, J.-L., Bernillon, J., Favre-Bonvin, J. & Arpin, N. (1981).** Mycosporins from *Ascochyta pisi*, *Cladosporium herbarum* and *Septoria nodorum*. *Phytochemistry* **20**, 2705–2707.
- Brown, A. D. (1976).** Microbial water stress. *Bacteriol Rev* **40**, 803–846.
- Brown, F. F., Sussman, I., Avron, M. & Degani, H. (1982).** NMR studies of glycerol permeability in lipid vesicles, erythrocytes and the alga *Dunaliella*. *Biochim Biophys Acta* **690**, 165–173.
- Butinar, L., Sonjak, S., Zalar, P., Plemenitaš, A. & Gunde-Cimerman, N. (2005).** Population dynamics of melanized halophilic fungi in solar salterns. *Botanica Marina* **48**, 73–79.
- Cantrell, S. A., Casillas-Martinez, L. & Molina, M. (2006).** Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. *Mycol Res* **110**, 962–970.
- Davis, D. J., Burlak, C. & Money, N. P. (2000).** Osmotic pressure of fungal compatible osmolytes. *Mycol Res* **104**, 800–804.
- de Jong, J. C., McCormack, B. J., Smirnoff, N. & Talbot, N. J. (1997).** Glycerol generates turgor in rice blast. *Nature* **389**, 244–245.
- Diano, A., Bekker-Jensen, S., Dynesen, J. & Nielsen, J. (2006).** Polyol synthesis in *Aspergillus niger*: influence of oxygen availability, carbon and nitrogen sources on the metabolism. *Biotechnol Bioeng* **94**, 899–908.
- Galinski, E. A. (1995).** Osmoadaptation in bacteria. *Adv Microb Physiol* **37**, 272–328.
- Galinski, E. A. & Herzog, R. M. (1990).** The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*). *Arch Microbiol* **153**, 607–613.
- Galinski, E. A. & Trüper, H. G. (1994).** Microbial behaviour in salt-stressed ecosystems. *FEMS Microbiol Rev* **15**, 95–108.
- Gimmler, H. & Hartung, W. (1988).** Low permeability of the plasma membrane of *Dunaliella parva* for solutes. *J Plant Physiol* **133**, 165–172.
- Gunde-Cimerman, N., Zalar, P., de Hoog, S. & Plemenitaš, A. (2000).** Hypersaline waters in salterns – natural ecological niches for halophilic black yeasts. *FEMS Microbiol Ecol* **32**, 235–240.
- Hohmann, S. (2002).** Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* **66**, 300–372.
- Jacobson, E. S. & Ikeda, R. (2005).** Effect of melanization upon porosity of the cryptococcal cell wall. *Med Mycol* **43**, 327–333.
- Kayingo, G., Kilian, S. G. & Prior, B. A. (2001).** Conservation and release of osmolytes by yeasts during hypo-osmotic stress. *Arch Microbiol* **177**, 29–35.
- Kim, S.-Y., Lee, K.-H., Kim, J.-H. & Oh, D.-K. (1997).** Erythritol production by controlling osmotic pressure in *Trigonopsis variabilis*. *Biotechnol Lett* **19**, 727–729.
- Klipp, E., Nordlander, B., Kruger, R., Gennemark, P. & Hohmann, S. (2005).** Integrative model of the response of yeast to osmotic shock. *Nat Biotechnol* **23**, 975–982.
- Kogej, T., Wheeler, M. H., Lanišnik Rižner, T. & Gunde-Cimerman, N. (2004).** Evidence for 1,8-dihydroxynaphthalene melanin in three halophilic black yeasts grown under saline and non-saline conditions. *FEMS Microbiol Lett* **232**, 203–209.
- Kogej, T., Ramos, J., Plemenitaš, A. & Gunde-Cimerman, N. (2005).** The halophilic fungus *Hortaea werneckii* and the halotolerant fungus *Aureobasidium pullulans* maintain low intracellular cation concentrations in hypersaline environments. *Appl Environ Microbiol* **71**, 6600–6605.
- Kogej, T., Gorbushina, A. A. & Gunde-Cimerman, N. (2006a).** Hypersaline conditions induce changes in cell-wall melanization and colony structure in a halophilic and a xerophilic black yeast species of the genus *Trimmatostroma*. *Mycol Res* **110**, 713–724.
- Kogej, T., Gostinčar, C., Volkmann, M., Gorbushina, A. A. & Gunde-Cimerman, N. (2006b).** Mycosporines in extremophilic fungi – novel complementary osmolytes? *Environ Chem* **3**, 105–110.
- Libkind, D., Pérez, P., Sommaruga, R., Diéguez, M. d. C., Ferraro, M., Brizzio, S., Zagarese, H. & van Broock, M. (2004).** Constitutive and UV-inducible synthesis of photoprotective compounds (carotenoids and mycosporines) by freshwater yeasts. *Photochem Photobiol Sci* **3**, 281–286.
- Luyten, K., Albertyn, J., Skibbe, W. F., Prior, B. A., Ramos, J., Thevelein, J. M. & Hohmann, S. (1995).** Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. *EMBO J* **14**, 1360–1371.
- Mager, W. H. & Siderius, M. (2002).** Novel insights into the osmotic stress response of yeast. *FEMS Yeast Res* **2**, 251–257.
- Méjanelle, L., López, J. F., Gunde-Cimerman, N. & Grimalt, J. O. (2000).** Sterols of melanized fungi from hypersaline environments. *Org Geochem* **31**, 1031–1040.
- Nesci, A., Etcheverry, M. & Magan, N. (2004).** Osmotic and matrix potential effects on growth, sugar alcohol and sugar accumulation by *Aspergillus* section *Flavi* strains from Argentina. *J Appl Microbiol* **96**, 965–972.
- Nobre, M. F. & da Costa, M. S. (1985).** The accumulation of polyols by the yeast *Debaryomyces hansenii* in response to water stress. *Can J Microbiol* **31**, 1061–1064.
- Oren, A. (1997).** Mycosporine-like amino acids as osmotic solutes in a community of halophilic cyanobacteria. *Geomicrobiol J* **14**, 231–240.
- Oren, A. (1999).** Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev* **63**, 334–348.
- Oren, A. (2002).** *Halophilic Microorganisms and their Environments*. Dordrecht: Kluwer Academic Publishers.
- Oren, A. & Gunde-Cimerman, N. (2007).** Mycosporines and mycosporine-like amino acids: UV protectants or multipurpose secondary metabolites? *FEMS Microbiol Lett* **269**, 1–10.
- Petrović, U., Gunde-Cimerman, N. & Plemenitaš, A. (2002).** Cellular responses to environmental salinity in the halophilic black yeast *Hortaea werneckii*. *Mol Microbiol* **45**, 665–672.
- Pfyffer, G. E., Pfyffer, B. U. & Rast, D. M. (1986).** The polyol pattern, chemotaxonomy, and phylogeny of the fungi. *Sydowia* **39**, 160–201.
- Plemenitaš, A. & Gunde-Cimerman, N. (2005).** Cellular responses in the halophilic black yeast *Hortaea werneckii* to high environmental salinity. In *Adaptation to Life at High Salt Concentrations in Archaea, Bacteria, and Eukarya*, pp. 453–470. Edited by N. Gunde-Cimerman, A. Oren & A. Plemenitaš. Dordrecht: Springer.

- Prista, C., Almagro, A., Loureiro-Dias, M. C. & Ramos, J. (1997).** Physiological basis for the high salt tolerance of *Debaryomyces hansenii*. *Appl Environ Microbiol* **63**, 4005–4009.
- Sheffer, M., Fried, A., Gottlieb, H., Tietz, A. & Avron, M. (1986).** Lipid composition of the plasma-membrane of the halotolerant alga *Dunaliella salina*. *Biochim Biophys Acta* **857**, 165–172.
- Turk, M., Méjanelle, L., Šentjurc, M., Grimalt, J. O., Gunde-Cimerman, N. & Plemenitaš, A. (2004).** Salt-induced changes in lipid composition and membrane fluidity of halophilic yeast-like melanized fungi. *Extremophiles* **8**, 53–61.
- Van Eck, J. H., Prior, B. A. & Brandt, E. V. (1989).** Accumulation of polyhydroxy alcohols by *Hansenula anomala* in response to water stress. *J Gen Microbiol* **135**, 3505–3513.
- Voegele, R. T., Hahn, M., Lohaus, G., Link, T., Heiser, I. & Mendgen, K. (2005).** Possible roles for mannitol and mannitol dehydrogenase in the biotrophic plant pathogen *Uromyces fabae*. *Plant Physiol* **137**, 190–198.
- Volkman, M. & Gorbushina, A. A. (2006).** A broadly applicable method for extraction and characterization of mycosporines and mycosporine-like amino acids of terrestrial, marine and freshwater origin. *FEMS Microbiol Lett* **255**, 286–295.
- Witteveen, C. F. B. & Visser, J. (1995).** Polyol pools in *Aspergillus niger*. *FEMS Microbiol Lett* **134**, 57–62.
- Yancey, P. H. (2005).** Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J Exp Biol* **208**, 2819–2830.

---

Edited by: N. L. Glass