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The water relations of growth and polyhydroxy alcohol production by ascomycetous yeasts

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The response of 31 ascomycetous yeasts to a reduction in water activity (a_w) adjusted with D-glucose or NaCl was investigated. The growth of most yeasts was more tolerant to glucose than to NaCl at equivalent a_w. *Zygosaccharomyces rouxii* was the most osmotolerant yeast examined. Natural abundance ^{13}C-NMR spectroscopy and HPLC analyses of eight yeasts indicated that glycerol and arabitol or mannitol were accumulated intracellularly in response to a_w reduction. *Pichia sorbitophila, Candida cacaoi, Candida magnoliae* and *Zygosaccharomyces bisporus* responded to reduced a_w by a decrease in specific growth rate and cell volume, and an accumulation of glycerol. The other polyol accumulated did not increase in concentration with a_w reduction to the same degree as glycerol. A polyol concentration ratio (intra/extracellular) as high as 800-fold was attained across the membrane. Greater amounts of polyols were produced at equivalent a_w values when adjusted with glucose than with NaCl. The ability to accumulate high concentrations of polyols appears to be the most important criterion in determining osmotolerance.

Introduction

Low molecular mass compounds are accumulated intracellularly in most organisms when exposed to osmotic stress to equilibrate the cytoplasmic water activity (a_w) with the a_w of the surrounding environment (Yancey *et al.*, 1982; Csonka, 1989). The main solutes accumulated in yeasts exposed to osmotic stress are polyhydroxy alcohols (polyols) such as glycerol, D-arabitol, D-mannitol and *meso*-erythritol (Spencer & Spencer, 1978), and are compatible with metabolic activity (known as compatible solutes; Brown, 1978). The polyol accumulated is related to the yeast species, the growth phase of the yeast (Nobre & da Costa, 1985) and the carbon source used for growth (Van Eck *et al.*, 1989). The terms 'osmophilic' and 'osmotolerant' have been widely used to describe the water relations of yeasts. Whereas relatively few yeasts can be described as having a requirement for reduced a_w (i.e. osmophilic; Jermini & Schmidt-Lorenz, 1987), many yeasts are described in the taxonomic literature as osmotolerant by virtue of their ability to grow in the presence of 60% glucose (Barnett *et al.*, 1983). The distinction between osmotolerant and non-osmotolerant yeasts, however, is poorly understood on a genetic and physiological basis, and the relationship of polyol accumulation to osmotolerance has only been described in a few yeasts (Adler *et al.*, 1985; Edgley & Brown, 1978; Nobre & da Costa, 1985; Reed *et al.*, 1987; Van Eck *et al.*, 1989). The purpose of this study was to investigate the water relations of a wide range of yeasts in terms of their growth requirements, cell volume, types of solutes accumulated and the characteristics of the accumulation process in a selected group of yeasts. Most of the yeasts investigated were chosen on the basis of their frequent occurrence in high-sugar and high-salt foods (Barnett *et al.*, 1983).

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Methods

Organisms and media. Yeasts were obtained from J. P. van der Walt of the Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa, the Centraalbureau voor Schimmelcultures (CBS), Delft, the Netherlands, C. P. Kurtzman, Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA and the American Type Culture Collection (ATCC), Rockville, Maryland, USA (details in legend to Fig. 1). The cultures were maintained on YM agar slants (Wickerham, 1951). All growth experiments were conducted in yeast nitrogen base (YNB) medium (Difco; 6·7 g l^{-1}) containing 10 g D-glucose l^{-1}.

Growth studies. The inoculum for each yeast was prepared by seeding glucose-YNB medium (100 ml) in 500 ml Erlenmeyer flasks and culturing with vigorous agitation on a rotary shaker (180 r.p.m.) at 30 °C until late exponential growth phase. The inoculum (0·5 ml) was used to seed glucose-YNB medium (10 ml) in tubes. The a_w of the medium was adjusted with either D-glucose (Norrish, 1966) or NaCl (Robinson & Stokes, 1959). The tubes were incubated at 30 °C with

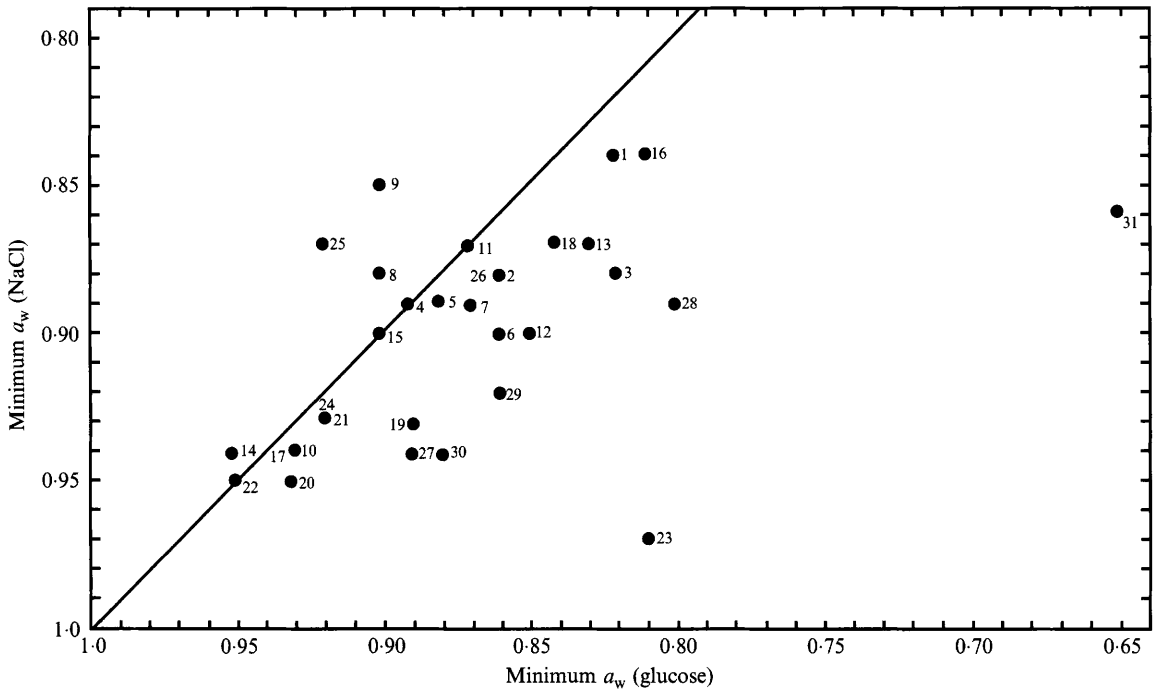

Fig. 1. The minimum a_w (NaCl or D-glucose) as determined in tubes for the growth of the following ascomycetous yeasts in glucose-YNB medium: 1, *Candida cacaoi* CBS 2020; 2, *Candida homilentoma* CSIR Y917; 3, *Candida magnoliae* NRRL YB4226; 4, *Candida silvicultrix* CSIR Y481; 5, *Candida tropicalis* CBS 94; 6, *Citromyces matritensis* CSIR Y159; 7, *Debaryomyces castellii* CSIR Y229; 8, *Debaryomyces hansenii* CSIR Y953; 9, *Geotrichium terrestre* CSIR Y803; 10, *Hansenula anomala* CSIR Y207; 11, *Hansenula ciferrii* CSIR Y804; 12, *Hansenula jadinii* CSIR Y227; 13, *Hansenula sydowiorum* CSIR Y463; 14, *Lipomyces kononenkoae* CSIR Y353; 15, *Pichia farinosa* CSIR Y226; 16, *Pichia membraneaefaciens* CSIR Y35; 17, *Pichia sorbitophila* CSIR Y170; 18, *Pichia stipitis* CSIR Y567; 19, *Saccharomyces cerevisiae* ATCC 4126; 20, *Saccharomyces dairensis* CSIR Y844; 21, *Saccharomyces exiguus* CSIR Y847; 22, *Schizosaccharomyces octosporus* CSIR Y934; 23, *Schizosaccharomyces pombe* CSIR Y457; 24, *Schwanniomyces occidentalis* CSIR Y936; 25, *Stephanoascus ciferrii* CSIR Y487; 26, *Wingea robertsiae* CSIR Y446; 27, *Zygosaccharomyces bailii* CSIR Y126; 28, *Zygosaccharomyces bisporus* CSIR Y849; 29, *Zygosaccharomyces fermentii* CSIR Y860; 30, *Zygosaccharomyces florentinus* CSIR Y576; 31, *Zygosaccharomyces rouxii* CSIR Y364. The diagonal line represents the division between yeasts with greater NaCl tolerance (upper left) and those with greater glucose tolerance (lower right).

weekly agitation on a Vortex mixer. Growth was monitored at 640 nm with a Klett–Summerson colorimeter. Three doublings in absorbance were taken to represent growth at the different a_w values.

Growth of and polyol accumulation (as identified by ^{13}C-NMR spectroscopy and quantified by HPLC) by yeasts at reduced a_w values was examined by preparing inocula in 50 ml glucose-YNB medium (250 ml Erlenmeyer flasks) as described above. These cultures (6·5 ml) were used to inoculate glucose-YNB (130 ml) in 500 ml Erlenmeyer flasks equipped with side-arm cuvettes. The cultures were incubated under the same conditions as the inocula. Growth was monitored with a Klett–Summerson colorimeter at 640 nm and the contents of the flasks were harvested for analyses by centrifugation (15000 ***g*** for 10 min) during the late exponential growth phase.

Analytical methods. Methods for the determination of biomass, cell volume, polyol concentration, calculation of the intracellular a_w and the identification of accumulated solutes by ^{13}C-NMR spectroscopy were described previously (Van Eck *et al.*, 1989).

Results

Minimum a_w for growth

The minimum a_w values for growth of the yeasts are shown in Fig. 1. Of the 31 yeasts evaluated, 23 tolerated glucose better than NaCl at equivalent a_w values, whereas four yeasts were more NaCl-tolerant and four were equitolerant. Genera such as *Pichia*, *Saccharomyces*, *Schizosaccharomyces* and *Zygosaccharomyces* were more glucose-tolerant than NaCl-tolerant at equivalent a_w values. Members of the genera *Zygosaccharomyces*, *Pichia*, *Candida*, *Hansenula* and *Geotrichium* were the most tolerant of the 13 genera evaluated. *Zygosaccharomyces rouxii* was exceptionally more osmotolerant than the other yeasts.

Identification of solutes accumulated by yeasts grown at 0·998 and 0·95 a_w values (glucose or NaCl)

The ^{13}C-NMR spectra of *Candida magnoliae*, *Pichia sorbitophila* and *Saccharomyces cerevisiae* grown at 0·998 a_w indicated that mannitol and glycerol, arabitol and glycerol, and glycerol, respectively, were the main polyols accumulated (Fig. 2) with a large number of other compounds also present in significant concentrations (scans between 0 and 60, and 80 and 200 p.p.m.

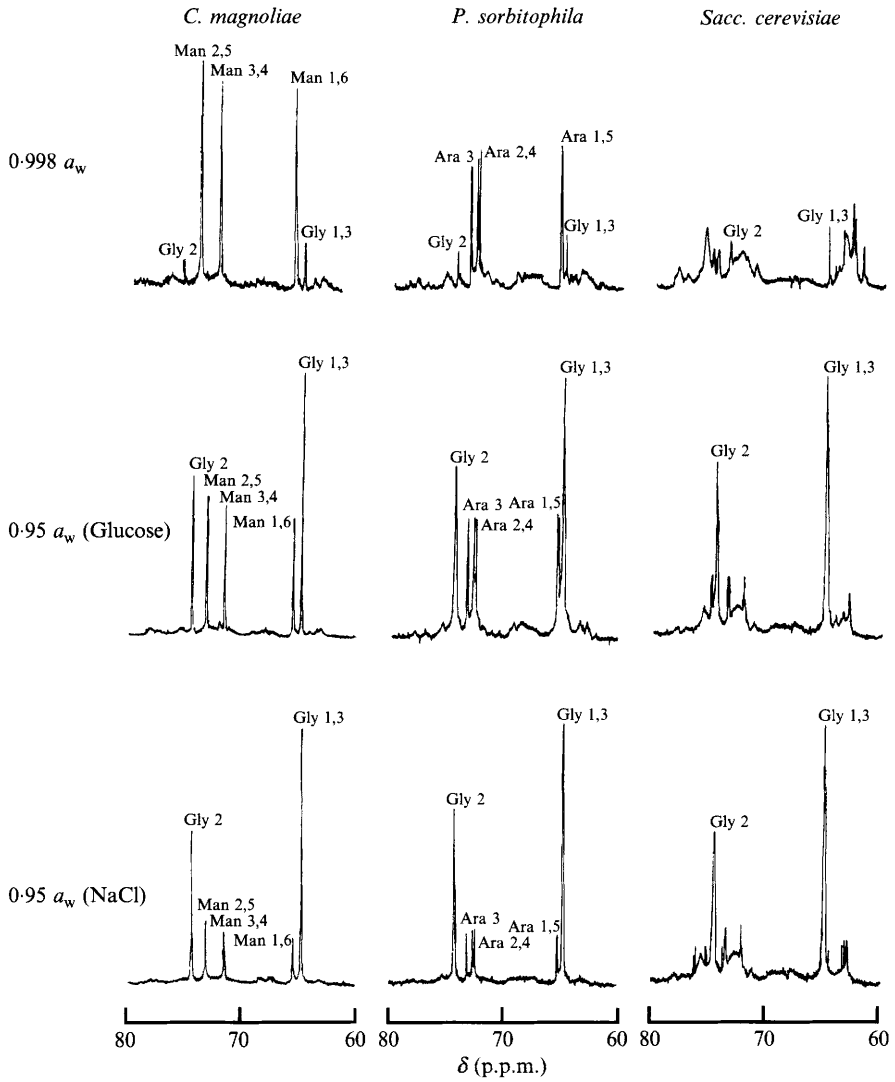

Fig. 2. Natural abundance ^{13}C-NMR scans of *C. magnoliae*, *P. sorbitophila* and *Sacc. cerevisiae* washed cells grown at 0·998 and 0·95 a_w (NaCl or D-glucose) in glucose-YNB medium in shake flasks. Ara, arabitol; Gly, glycerol; Man, mannitol.

not shown). When the a_w of the medium was reduced to 0·95 with glucose or NaCl, the peaks representing glycerol in all three yeasts increased in significance relative to the other peaks. However, the intensities of the spectra of each yeast are not comparable because of differences in gain. The peaks representing mannitol and arabitol found respectively in *C. magnoliae* and *P. sorbitophila* are apparently not regulated by osmotic stress. *Sacc. cerevisiae* under osmotic stress failed to accumulate another polyol. No other significant peaks were observed in scans between 0 and 200 p.p.m. (data not shown, except between 60 and 80 p.p.m.). ^{13}C-NMR scans of cultures of *Candida cacaoi*, *Candida silvicultrix*, *Debaryomyces hansenii*, *Zygosaccharomyces bisporus* and *Z. rouxii* grown at 0·998 a_w and at 0·95 a_w (NaCl or glucose) revealed that the only compounds osmotically regulated were glycerol and arabitol (data not shown).

Specific growth rate and cell volume at reduced a_w

When the a_w was reduced from 0·998 to 0·92 with either glucose or NaCl, the specific growth rates of *C. cacaoi* (Fig. 3), *C. magnoliae* (Fig. 4), *P. sorbitophila* (Fig. 5) and *Z. bisporus* (Fig. 6) decreased, which suggests that these yeasts are osmotolerant rather than osmophilic. The cell volume of these yeasts was also smaller when grown at reduced a_w values (Figs 3–6), indicating that volume regulation is an important mechanism of adaption by yeasts to reduced a_w.

Polyol accumulation at reduced a_w

Reduction in the a_w of the growth medium with glucose or NaCl resulted in a marked increase in the intracellular glycerol concentration of the four yeasts (Figs 3–6) and at 0·92 a_w, the glycerol concentration varied between 150

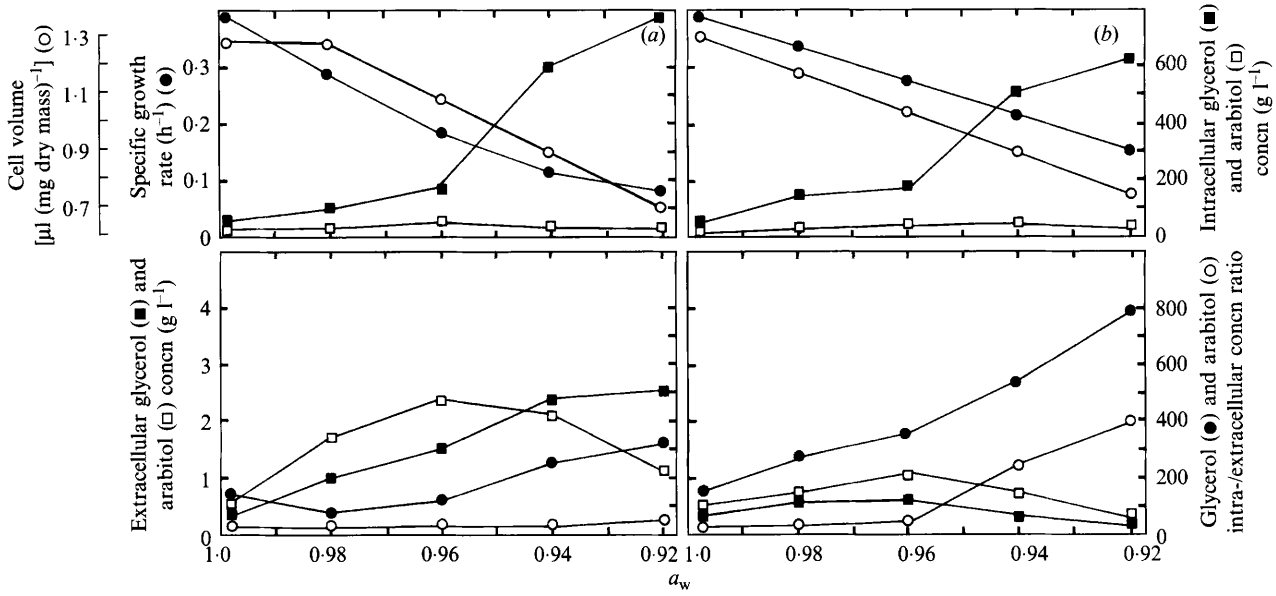

Fig. 3. Response of growth and polyol production by *C. cacaoi* in shake flasks containing glucose-YNB medium at 30 °C to water reduction with D-glucose (*a*) and NaCl (*b*).

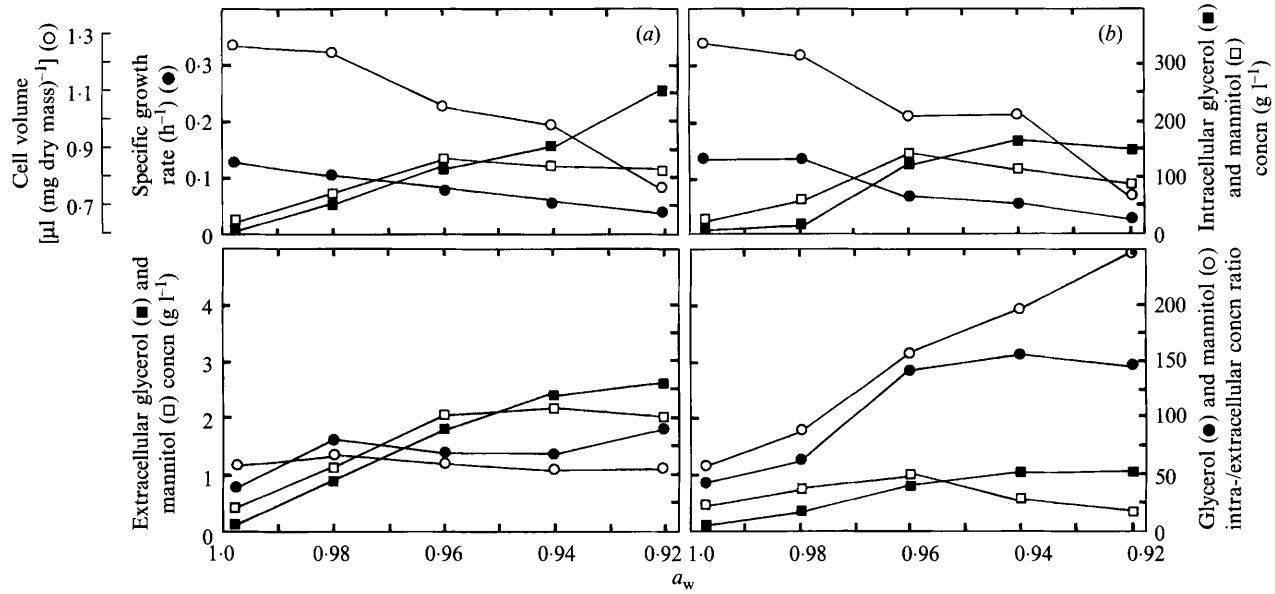

Fig. 4. Response of growth and polyol production by *C. magnoliae* in shake flasks containing glucose-YNB medium at 30 °C to water reduction with D-glucose (*a*) and NaCl (*b*).

and 760 g l^{-1}. The intracellular concentrations of arabitol (*C. cacaoi*, *P. sorbitophila* or *Z. bisporus*) or mannitol (*C. magnoliae*) increased only slightly or not at all when the a_w of the growth medium was reduced, which suggests that these polyols are not as osmotically responsive as glycerol.

Extracellular polyol concentrations increased with a reduction of a_w (Figs 3–6) although in a number of instances, the concentrations decreased at a_w values less than 0·96 which suggests that a mechanism may operate to retard the leakage of the polyols from the cell. In contrast to marked differences in the intracellular concentrations of glycerol and the other polyols at reduced a_w, the polyol concentrations in the medium

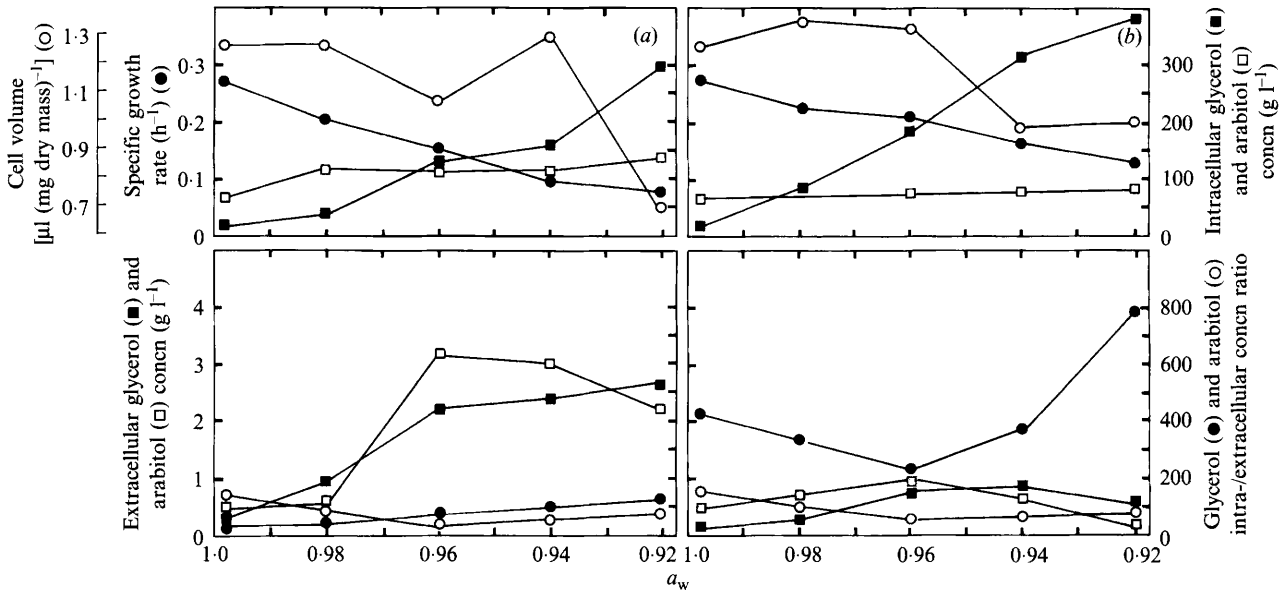

Fig. 5. Response of growth and polyol production by *P. sorbitophila* in shake flasks containing glucose-YNB medium at 30 °C to water reduction with D-glucose (*a*) and NaCl (*b*).

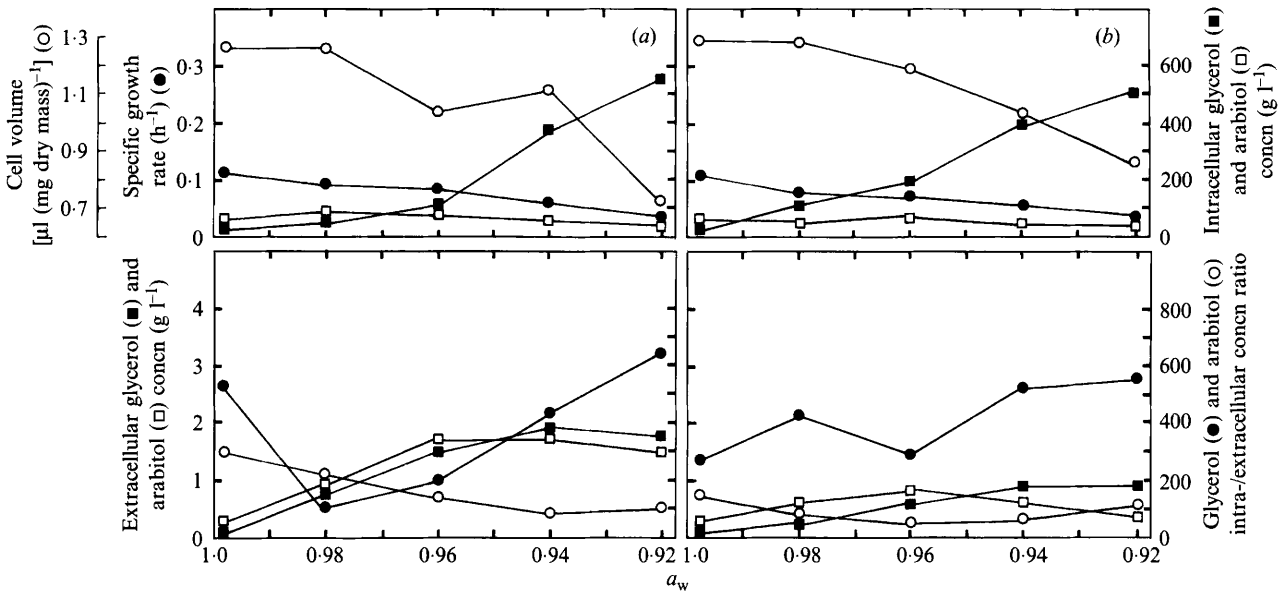

Fig. 6. Response of growth and polyol production by *Z. bisporus* in shake flasks containing glucose-YNB medium at 30 °C to water reduction with D-glucose (*a*) and NaCl (*b*).

were fairly similar. In general, the extracellular polyol concentrations were much greater when the a_w was adjusted with glucose than with NaCl.

Polyol concentration ratio (intra-/extracellular)

In most instances, a reduction in the a_w to 0·92 resulted in an increase in the ratio of intracellular to extracellular glycerol concentrations (Figs 3–6) and at 0·92 a_w (NaCl), glycerol ratios as high as 800 were observed. In *C. cacaoi* (Fig. 3) and *P. sorbitophila* (Fig. 5) the glycerol ratios were greater in media adjusted with NaCl than with glucose. In *C. magnoliae* (Fig. 4) the reverse was observed, whereas in *Z. bisporus* (Fig. 6) no consistent pattern was found. The arabitol concentration ratio (or mannitol in *C. magnoliae*) increased only slightly or not at all, or in some instances decreased with a reduction of the a_w. When the a_w was adjusted with NaCl, the glycerol

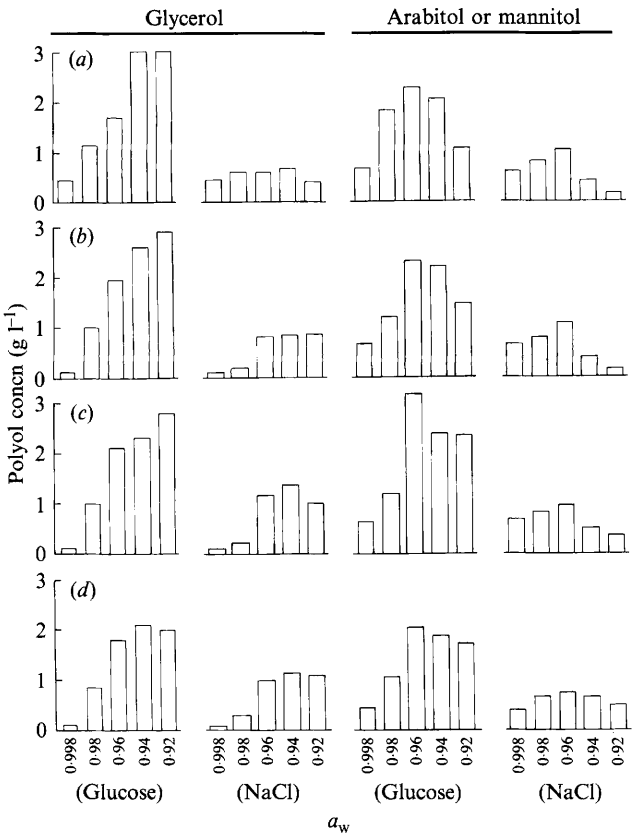

Fig. 7. Effect of a_w on the total production of polyols in shake flasks by (*a*) *C. cacaoi* (glycerol and arabitol), (*b*) *C. magnoliae* (glycerol and mannitol), (*c*) *P. sorbitophila* (glycerol and arabitol) and (*d*) *Z. bisporus* (glycerol and arabitol). The water activity of the glucose-YNB medium was adjusted with either D-glucose or NaCl.

ratio was greater than the ratio of the other polyols in all instances except in *C. magnoliae*.

Production of polyols

The total concentration of glycerol produced (intra- and extracellular) increased with reduction of a_w and was highest at 0·92 a_w (glucose) with the exception of *Z. bisporus* (Fig. 7*a*). In contrast, lower concentrations of glycerol were produced when the a_w was adjusted with NaCl. Calculations, however, showed that the percentage glycerol retained within the cell was, in most instances, greater when the a_w was adjusted with NaCl than with glucose. In general, the percentage retained decreased with a reduction in a_w. A notable exception was *C. cacaoi* which retained 70% of the total glycerol produced at 0·92 a_w (NaCl).

Arabitol (or mannitol in *C. magnoliae*) production was greater in media adjusted with glucose than with NaCl and attained a maximum concentration at 0·96 a_w (Fig. 7*b*). A marked reduction was observed at a_w values less than 0·96. Calculations of the percentage arabitol (or mannitol) retained intracellularly showed, in all instances, a decrease with a_w.

Discussion

The minimum a_w values for the 31 yeasts reported here are, in general, similar to values disclosed elsewhere for a limited number of ascomycetous yeasts (Tilbury, 1980; Tokuoka & Ishitani, 1991). The minimum a_w for the growth of *Z. rouxii* has frequently been reported. The minimum value of 0·65 a_w (glucose) found here is similar to 0·62 a_w (sucrose) and 0·65 a_w (glucose) reported by Restaino *et al.* (1983) and Tilbury (1980), respectively. Others have found that the minimum growth a_w for *Z. rouxii*, adjusted with glucose, fructose or sucrose, was much higher and may reflect differences between strains, carbon sources or medium composition (Bellinger & Larher, 1988; Jermini & Schmidt-Lorenz, 1987; Tokuoka & Ishitani, 1991). Comparison of the minimum growth a_w values (glucose or NaCl) reported here for *D. hansenii*, *Hansenula anomala*, *Pichia membraneafaciens*, *Sacc. cerevisiae*, *Schizosaccharomyces pombe*, *Candida tropicalis* and *Z. bisporus* with values determined by Tokuoka & Ishitani (1991) showed similar values in most cases with a few notable exceptions. The greater tolerance of some yeasts to glucose or NaCl can be related to their ecology. For example, salt-tolerant *D. hansenii* is found largely in marine environments and salt foods (Barnett *et al.*, 1983). Sugar-tolerant yeasts such as *Zygosaccharomyces bailii*, *Z. florentinus* and *Z. rouxii* are commonly isolated from foods containing high sugar concentrations (Barnett *et al.*, 1983). Interestingly, Tokuoka & Ishitani (1991) found that the *Z. rouxii* strains isolated from high-salt foods tolerated NaCl to a greater extent than those isolated from high-sugar foods. In all instances, however, the minimum growth a_w of these strains was lower when adjusted with glucose than with NaCl. The ability of some strains and species to tolerate higher NaCl concentrations could be due to the presence of a sodium extrusion pump (Hobot & Jennings, 1981; Norkrans & Kylin, 1969). Some yeasts evidently transport glycerol into their cells with sodium ions (Lucas *et al.*, 1990; Van Zyl *et al.*, 1990), yet may not possess an efficient mechanism to expel sodium ions.

^{13}C-NMR spectroscopy revealed that only glycerol, arabitol and mannitol were solutes whose concentrations were significantly increased during osmotic stress in ascomycetous yeasts. Glycerol was the major osmotically active solute found in the eight yeast species studied here as well as a total of 18 species investigated by Brown (1978), Reed *et al.* (1987), Meikle *et al.* (1991) and Bellinger & Lahrer (1988). Furthermore, glycerol is the principal osmolyte in some filamentous fungi, algae, insects, crustaceans and vertebrates which indicates that

there is a selective advantage to organisms using such low molecular mass solutes (Yancey *et al.*, 1982; Hocking & Norton, 1983). As an osmolyte, glycerol offers a number of advantages: high glycerol concentrations confer a remarkable degree of protection to enzymes and macromolecular structure (Brown, 1978), and modification of proteins is unnecessary in order to function in concentrated intracellular solutions. Furthermore, the solubility and viscosity of glycerol are hardly affected by increasing concentrations of this polyol (Chirife *et al.*, 1983, 1984).

With the exception of *Sacc. cerevisiae*, ascomycetous yeasts were found to contain the polyols arabitol or mannitol. Intracellular accumulation of these solutes, however, does not appear to respond to osmotic stress to the same degree as observed with glycerol and their role in osmoregulation is uncertain. They may act as reserve compatible solutes as exhaustion of the energy source during the late exponential growth phase leads to the possible consumption of glycerol by *D. hansenii* (Nobre & da Costa, 1985) and *H. anomala* (Van Eck *et al.*, 1989). *Sacc. cerevisiae*, however, failed to produce a secondary polyol, yet the degree of osmotolerance of this yeast was not significantly less than other yeasts that produced two polyols. These secondary solutes, however, may be important in sustaining viability when a yeast is exposed to a sudden osmotic shock (Brown *et al.*, 1986; Van Zyl & Prior, 1990).

Accumulation of other solutes, such as amino acids and betaines, has often been observed in bacteria and plants in response to osmotic stress (Yancey *et al.*, 1982; Csonka, 1989). We failed to observe these solutes in the ^{13}C-NMR spectra of the yeasts investigated and it would appear that polyols, especially glycerol, are the only organic solutes used to regulate intracellular osmotic pressure (Meikle *et al.*, 1991).

The ability to retain a greater proportion of a constant total (intra- and extracellular) amount of glycerol intracellularly was proposed by Brown (1978) as an essential characteristic of an osmotolerant yeast such as *Z. rouxii*, whereas a non-osmotolerant yeast such as *Sacc. cerevisiae* produces increasing amounts of glycerol and retains intracellularly a decreasing proportion of the total. The four yeasts (*C. cacaoi*, *C. magnoliae*, *P. sorbitophila* and *Z. bisporus*) were found to have a minimum growth a_w value adjusted with glucose, of less than 0·84 which, according to the taxonomic literature (Barnett *et al.*, 1983), would classify them as osmotolerant yeasts. Their behaviour in producing and accumulating glycerol was, however, more similar to that observed with *Sacc. cerevisiae* (Brown, 1978) which suggests that the exceptional osmotolerance and different behaviour of *Z. rouxii* may be unique amongst the yeasts. Calculations of the intracellular a_w from the glycerol concentration (Weast, 1984) indicated that in most instances sufficient glycerol was accumulated by the four yeasts to enable the intra- and extracellular a_w to attain an equilibrium. Apparently, these yeasts are osmotolerant by virtue of their ability to produce increasing amounts of glycerol with decreasing a_w rather than to retain a greater proportion of a constant amount of glycerol. Furthermore, the current division of yeasts into osmotolerant and non-osmotolerant has no clear physiological basis.

The increasing ratio (intra-/extracellular concentration) of glycerol with a decrease in a_w indicates that the permeability of the plasma membrane is involved in the water relations of yeasts (Brown, 1978). During osmotic stress, the permeability of the yeast membrane to glycerol and other solutes may be reduced by changes in the phospholipid composition (Watanabe & Takakuwa, 1984; Tunblad-Johansson & Adler, 1987) thus allowing solute accumulation. Alternatively, yeasts may maintain high intracellular concentrations by means of active transport of glycerol. Active transport mechanisms regulated by osmotic stress have been described in *D. hansenii* (Adler *et al.*, 1985; Lucas *et al.*, 1990) and *Z. rouxii* (Van Zyl *et al.*, 1990) which enable glycerol to be accumulated intracellularly up to 5000-fold. Furthermore, the greater retention of glycerol as observed here when the a_w was adjusted with NaCl than with glucose could be related to the use of a sodium gradient as the driving force to maintain the glycerol gradient.

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References

ADLER, L., BLOMBERG, A. & NILSSON, A. (1985). Glycerol metabolism and osmoregulation in the salt-tolerant yeast *Debaryomyces hansenii*. *Journal of Bacteriology* **162**, 300–306.

BARNETT, J. A., PAYNE, R. W. & YARROW, D. (1983). *Yeasts: Characteristics and Identification.* Cambridge & New York: Cambridge University Press.

BELLINGER, Y. & LARHER, F. (1988). A ^{13}C comparative nuclear magnetic resonance study of organic solute production and excretion by the yeasts *Hansenula anomala* and *Saccharomyces cerevisiae* in saline media. *Canadian Journal of Microbiology* **34**, 605–612.

BROWN, A. D. (1978). Compatible solutes and extreme water stress in eukaryotic microorganisms. *Advances in Microbial Physiology* **17**, 181–242.

BROWN, A. D., MACKENZIE, K. F. & SINGH, K. K. (1986). Selected aspects of microbial osmoregulation. *FEMS Microbiology Reviews* **39**, 31–36.

CHIRIFE, J., ALZAMORA, S. M. & FERRO FONTAN, C. F. (1983). Microbial growth at reduced water activities: studies of a_w prediction in solutions of compatible solutes. *Journal of Applied Bacteriology* **54**, 339–343.

CHIRIFE, J., FAVETTO, G. & FERRO FONTAN, C. F. (1984). Microbial growth at reduced water activities: some physicochemical properties of compatible solutes. *Journal of Applied Bacteriology* **56**, 259–268.

CSONKA, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews* **53**, 121–147.

EDGLEY, M. & BROWN, A. D. (1978). Response of xerotolerant and non-tolerant yeasts to water stress. *Journal of General Microbiology* **104**, 343–345.

HOBOT, J. A. & JENNINGS, D. H. (1981). Growth of *Debaryomyces hansenii* and *Saccharomyces cerevisiae* in relation to pH and salinity. *Experimental Mycology* **5**, 217–228.

HOCKING, A. D. & NORTON, R. S. (1983). Natural-abundance ^{13}C nuclear magnetic resonance studies on the internal solutes of xerophilic fungi. *Journal of General Microbiology* **129**, 2915–2925.

JERMINI, M. F. G. & SCHMIDT-LORENZ, W. (1987). Growth of osmotolerant yeasts at different water activity values. *Journal of Food Protection* **50**, 404–410.

LUCAS, C., DA COSTA, M. & VAN UDEN, N. (1990). Osmoregulatory active sodium–glycerol co-transport in the halotolerant yeast *Debaryomyces hansenii*. *Yeast* **6**, 187–191.

MEIKLE, A. J., CHUDEK, J. A., REED, R. H. & GADD, G. M. (1991). Natural abundance ^{13}C-nuclear magnetic resonance spectroscopic analysis of acyclic polyol and trehalose accumulation by several yeast species in response to salt stress. *FEMS Microbiology Letters* **82**, 163–168.

NOBRE, M. F. & DA COSTA, M. S. (1985). Factors favouring the accumulation of arabitol in the yeast *Debaryomyces hansenii*. *Canadian Journal of Microbiology* **31**, 467–471.

NORKRANS, B. & KYLIN, A. (1969). Regulation of potassium to sodium ratio and the osmotic potential in relation to salt tolerance in yeasts. *Journal of Bacteriology* **100**, 836–845.

NORRISH, R. S. (1966). An equation for the activity coefficients and equilibrium relative humidities of water in confectionary syrups. *Journal of Food Technology* **1**, 25–39.

REED, R. H., CHUDEK, J. A., FOSTER, R. & GADD, G. M. (1987). Osmotic significance of glycerol accumulation in exponentially growing yeasts. *Applied and Environmental Microbiology* **53**, 2119–2123.

RESTAINO, L., BILLS, S., TSCHERNEFF, K. & LENOVICH, L. M. (1983). Growth characteristics of *Zygosaccharomyces rouxii* isolated from chocolate syrup. *Applied and Environmental Microbiology* **45**, 1614–1621.

ROBINSON, R. A. & STOKES, R. H. (1959). *Electrolyte Solutions*. New York: Academic Press.

SPENCER, J. F. T. & SPENCER, D. M. (1978). Production of polyhydroxy alcohols by osmotolerant yeasts. In *Economic Microbiology*, vol. 2, pp. 393–425. Edited by A. H. Rose. London: Academic Press.

TILBURY, R. H. (1980). Xerotolerant (osmophilic) yeasts. In *Biology and Activities of Yeasts*, pp. 152–179. Edited by F. A. Skinner, S. M. Passmore & R. R. Davenport. London: Academic Press.

TOKUOKA, K. & ISHITANI, T. (1991). Minimum water activities for the growth of yeasts isolated from high-sugar foods. *Journal of General and Applied Microbiology* **37**, 111–119.

TUNBLAD-JOHANSSON, I. & ADLER, L. (1987). Effects of sodium chloride concentration on phospholipid fatty acid composition of yeasts differing in osmotolerance. *FEMS Microbiology Letters* **43**, 275–278.

VAN ECK, J. H., PRIOR, B. A. & BRANDT, E. V. (1989). Accumulation of polyhydroxy alcohols by *Hansenula anomala* in response to water stress. *Journal of General Microbiology* **135**, 3505–3513.

VAN ZYL, P. J. & PRIOR, B. A. (1990). Adaptation of *Zygosaccharomyces rouxii* to changes in water activity in transient continuous culture. *Biotechnology Letters* **12**, 361–366.

VAN ZYL, P. J., KILIAN, S. G. & PRIOR, B. A. (1990). The role of an active transport mechanism in glycerol accumulation during osmoregulation by *Zygosaccharomyces rouxii*. *Applied Microbiology and Biotechnology* **34**, 231–235.

WATANABE, Y. & TAKAKUWA, M. (1984). Effects of sodium chloride on lipid composition of *Saccharomyces rouxii*. *Agricultural and Biological Chemistry* **48**, 2415–2422.

WEAST, R. C. (1984). In *CRC Handbook of Chemistry and Physics*, 65th edition, p. D235 Edited by R. C. Weast. Boca Raton: CRC Press.

WICKERHAM, L. S. (1951). Taxonomy of yeasts. *Technical Bulletin* no. 1029. Washington, DC: United States Department of Agriculture.

YANCEY, P. H., CLARK, M. E., HAND, S. C., BOWLUS, R. D. & SOMERO, G. N. (1982). Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214–1222.