Natural-abundance ^{13}C Nuclear Magnetic Resonance Studies on the Internal Solutes of Xerophilic Fungi

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(Received 16 March 1983)

Natural-abundance ^{13}C nuclear magnetic resonance spectroscopy was used to study the patterns of accumulation of osmotically active internal solutes in five different fungi. Four xerophilic fungi (*Penicillium janczewskii*, *Eurotium chevalieri*, *Xeromyces bisporus* and *Wallemia sebi*), and one non-xerophilic fungal species (*P. digitatum*) were grown at three different water activities (a_w) on media containing sorbitol, glucose/fructose or NaCl as the controlling solute. Under all conditions studied, the major internal solutes detected in aqueous ethanol extracts of these fungi were simple polyhydric alcohols: glycerol, erythritol, arabitol and mannitol. The most important osmoregulatory solute accumulated by all species was glycerol. On the sorbitol and the glucose/fructose media, all five fungi were able to accumulate glycerol. However, when NaCl was used to control a_w , only one species, *W. sebi*, was able to accumulate glycerol below 0.92 a_w . Significant quantities of the controlling solutes were also present in the extracts.

When intact mycelia of *P. janczewskii* were examined by NMR, resonances of all the major internal solutes were clearly discernible, although they were not as well resolved as those from the fungal extracts. Relaxation measurements showed that the solutes were relatively mobile inside the cells.

INTRODUCTION

The physiological and biochemical bases of the ability of some micro-organisms to grow under conditions of reduced water availability have been investigated by many workers. The mechanisms by which micro-organisms have adapted to growth at low water activity (a_w) have been shown to be diverse. Whatever the mechanism used, all organisms must be able to maintain a slight positive internal turgor pressure relative to their external environment.

Halophilic bacteria such as *Halobacterium* species, which require in excess of 3 M-NaCl in the growth medium, maintain their osmotic balance by accumulating high intracellular concentrations of K^+ (Christian & Waltho, 1962). Micro-algae use low molecular weight organic compounds such as sugars, polyols or polyol derivatives as osmoregulators. A number of species in the genus *Dunaliella* have been shown to use glycerol as the primary osmoregulatory solute (Craigie & McLachlan, 1964; Ben-Amotz & Avron, 1973; Borowitzka & Brown, 1974). Compounds which have been identified as osmoregulators in other species of algae include floridoside (Kauss, 1969), isofloridoside (Kauss, 1967), cyclohexanetetrol (Craigie, 1969), mannitol (Hellebust, 1976), mannisido-mannitol (Feige, 1972), sucrose (Wetherell, 1963), sorbitol and proline (Brown & Hellebust, 1978) and glucosylglycerol (Borowitzka *et al.*, 1980; Mackay *et al.*, 1983). The xerophilic yeast, *Saccharomyces rouxii* has been shown to accumulate glycerol efficiently in response to low external a_w (Brown, 1978), but the highly salt-tolerant yeast *Debaryomyces hansenii* accumulates K^+ intracellularly, as well as glycerol, as the salinity of the culture medium increases (Gustafsson & Norkrans, 1976).

There is little information about the osmoregulatory mechanisms in xerophilic filamentous

fungi. It has been assumed (Brown, 1978) that these micro-organisms use polyols, probably mainly glycerol, as osmoregulators in the same way as some yeasts and algae do. This has since been confirmed by Luard (1980) for at least two species, a xerophile, *Chrysosporium fastidium*, and a 'facultative' xerophile, *Penicillium chrysogenum*. Luard (1980) also showed that in the plant pathogen, *Phytophthora cinnamomi*, which is not xerophilic, proline was the major osmoregulatory solute.

Natural abundance ^{13}C nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for studying the role of organic solutes in osmoregulation, as all the major organic solutes in intact cells and in cell extracts can be identified and quantified (Norton, 1980). Although the technique is relatively insensitive (compared with ^1H NMR), and the natural abundance of ^{13}C is low (1.1%), solutes that are important in osmoregulation are generally present in concentrations sufficiently high to allow good spectra to be obtained in a relatively short time. Studies have been carried out on the marine mollusc *Tapes waiangi* (Norton, 1979; Norton & de Rome, 1980), in which taurine, betaine and glycine play a role in osmoregulation. The technique has also been used by Borowitzka *et al.* (1980) to study a cyanobacterium, *Synechococcus* sp., which was found to use glucosylglycerol as the major osmoregulatory solute. It has since been shown that glucosylglycerol is accumulated by all species of marine cyanobacteria in response to osmotic stress (Mackay *et al.*, 1983).

In this report, natural-abundance ^{13}C NMR spectroscopy has been used to investigate differences in the patterns of internal solutes accumulated by five fungi with differing water relations, over a range of water activities, and to observe the effects induced when different types of external solute are used to control a_w .

METHODS

Fungi. Four xerophilic and one non-xerophilic fungal species were chosen for study. The xerophilic species were: *Xeromyces bisporus*, an extreme xerophile which causes spoilage of high sugar substrates such as dried fruit, fruit cakes and confectionery; *Wallemia sebi*, which is more tolerant of high salt situations than other xerophilic fungi; *Eurotium chevalieri*, a common spoilage fungus in a wide range of low moisture foodstuffs; and *Penicillium janczewski*, one of the most xerophilic species in this ubiquitous genus (Pitt & Hocking, 1977; Hocking & Pitt, 1979). The non-xerophilic fungal species studied was *Penicillium digitatum* which is capable of growth only down to $0.90a_w$ (Hocking & Pitt, 1979). The following isolates were used: *Xeromyces bisporus* Fraser FRR 2347, from spoiled fruit cake, a_w 0.75, 1980; *Wallemia sebi* (Fries) v. Arx FRR 1471, from bread, 1973; *Eurotium chevalieri* Mangin FRR 547, from animal feed, 1970; *Penicillium janczewski* Zaleski FRR 1618, from soil, 1975; and *Penicillium digitatum* (Pers. ex Fr.) Sacc. FRR 1313, from orange, 1972. FRR denotes the culture collection of the CSIRO Division of Food Research, North Ryde, NSW, Australia.

Media. The basal medium was 1% yeast extract, 3% glucose, 0.4% KH_2PO_4 and 2% agar. The solutes used to adjust a_w were NaCl, sorbitol, or a mixture of equal weights of glucose and fructose. Analytical grade chemicals were used throughout. High, medium and low a_w values were chosen for study from the previously established growth range of each species in each solute system (Pitt & Hocking, 1977; Hocking & Pitt, 1979). For media containing NaCl as the controlling solute, the appropriate concentrations were calculated from the data of Robinson & Stokes (1955). A higher concentration of glucose (5%) was added to the NaCl media to partially offset the stringent conditions created by high numbers of sodium ions. Water activities ranged from 0.99 to 0.80. The formulae of Norrish (1966) were used to prepare glucose/fructose and sorbitol media with a_w values ranging from 0.99 to 0.75, and 0.99 to 0.80, respectively.

Cultivation. Glass Petri dishes, 140 mm in diameter, containing 50 ml medium, were overlaid with a circle of sterile dialysis membrane (Union Carbide Corp., Ill., U.S.A.). Approximately 0.1 ml of a suspension of spores of each fungus was dropped on to the membrane, and spread over the entire surface using a sterile glass spreader. The lids were replaced and the Petri dishes sealed with adhesive cellulose tape, and placed in polyethylene bags to minimize moisture loss during incubation. Plates were incubated at 25 °C, until growth was sufficient to permit extraction and analysis. At the high a_w values, this was usually achieved within 5 d, but at low a_w , incubation periods of up to 60 d were sometimes necessary.

For study of intact mycelia by NMR, *Penicillium janczewskii* was grown in submerged shake-flask culture, at 0.99, 0.92 and 0.85 a_w , in NaCl-based liquid media. Cultures were harvested by filtration through a Buchner funnel.

Extraction. The dialysis membrane was lifted off the agar, and the mycelial mat scraped off and weighed. Dry weights were determined on a separate sample, grown under the same conditions, by drying at 80 °C overnight, then at 70 °C for 6 h in a vacuum oven. Soluble carbohydrates were extracted from the fungal mycelium, initially

by the method of Maclean & Scott (1976). However, it was found that for ^{13}C NMR spectroscopy, chloroform clean-up and deionization of the extracts were unnecessary, so most samples were simply macerated in 80% (v/v) ethanol, boiled for 10 min, filtered through a Buchner funnel, and evaporated to near-dryness at 40–50 °C on a rotary evaporator. The extracts were taken up in the minimum amount of water and freeze-dried. Many extracts did not dry down to crystalline matter, but remained thick and syrupy. For NMR spectroscopy, the dried extracts were taken up in 2.0 ml D_2O , and the volume measured.

NMR spectra. Natural-abundance ^{13}C NMR spectra were recorded at 25.05 MHz on a Jeol FX-100 Q spectrometer operating in the pulsed Fourier transform mode with quadrature detection. Spinning sample tubes of 10 mm outer diameter were used. Fungal extracts were dissolved in D_2O , which served as an internal lock. The samples were pulsed with 100–800 60° radio-frequency pulses (16 μs pulse width), applied at 10.024 s intervals. A sweep width of 4000 Hz was used, and spectra were accumulated in 8192 data points. Exponential broadening of 1.1 Hz was applied before the data were Fourier transformed. Under these spectral accumulation conditions, none of the resonances of interest experienced any reduction in intensity due to saturation. Sample temperature was typically 25 °C, except for extracts of fungi grown at high NaCl concentrations. Residual salt in some of these extracts caused sample heating to temperatures of about 45 °C, presumably accompanied by detuning of the probe (Norton *et al.*, 1982), but these effects were not corrected for in considering peak intensities. Chemical shifts were measured digitally and are reported in parts per million downfield from external tetramethylsilane (TMS). A trace of dioxane (at 67.80 p.p.m. from external TMS) was used as an internal standard.

Spectra of intact fungal mycelia were obtained by gently packing filtered mycelia into the NMR tube so as to minimize air spaces (final packing densities were in the range 0.9–1.0 g ml⁻¹). Loosely packed mycelia surrounded by large pockets of air gave broader spectra. Spectra were recorded as above, except that 2000–4000 70° radio-frequency pulses were applied at 2.024 s intervals, and an external field-frequency lock was employed.

Spin-lattice relaxation times (T_1) were estimated from spectra recorded with a $(180^\circ-\tau-90^\circ-t)_n$ pulse sequence, using at least six τ intervals. Nuclear Overhauser enhancement (NOE) values were determined by comparison of integrated intensities in spectra recorded using complete proton decoupling with those recorded with decoupling gated on during data acquisition only, and off for a period 10-fold longer than the relevant T_1 values. All integrated intensities were measured digitally.

Solutes observed in spectra of fungal extracts were quantified by comparison of integrated intensities with corresponding values for known concentrations of the various solutes in aqueous solution. The intensities per unit carbon per mole for the individual solutes were essentially identical, confirming that all ^{13}C resonances experienced maximal NOE and were not saturated.

RESULTS

Intact mycelia

Natural-abundance ^{13}C NMR spectra of intact mycelia of *Penicillium janczewskii* grown in NaCl media in submerged culture are shown in Fig. 1. Even at the highest a_w (0.99, Fig. 1*a*), the major resonances observed were those from the polyols glycerol, erythritol and mannitol. The resonances of glycerol and erythritol were not resolved. Other peaks arose from the methylene carbons of fatty acids (30–31 p.p.m.), the olefinic carbons of unsaturated fatty acids (129–131 p.p.m.) and glucose. The peak at 54.5 p.p.m. may be due to *N*-trimethyl groups of phospholipids (Rosenthal & Fendler, 1976), although solutes such as glycine-betaine (Norton, 1979), could also contribute to this resonance. If this peak did arise from phospholipids, it would be likely that the resonances arose from membrane lipids as well as triglycerides.

At 0.92 a_w (Fig. 1*b*) the glycerol resonances increased relative to those of erythritol, mannitol and fatty acids, whilst at 0.85 a_w glycerol resonances dominated the spectrum (Fig. 1*c*).

The observation of relatively sharp resonances from glycerol, erythritol and mannitol suggests that these solutes enjoy considerable motional freedom within the intact mycelia (Norton, 1980). This is supported by spin-lattice relaxation time (T_1) values for intact mycelia grown at 0.99 a_w . Estimated T_1 values for the glycerol/erythritol peaks at 63.8 and 73.2 p.p.m. were 0.4 and 0.5 s, respectively, and for C-1/C-6, C-2/C-5 and C-3/C-4 of mannitol 0.4, 0.4 and 0.5 s, respectively, with an estimated experimental error of 20%. All resonances had the full NOE (average 2.84), indicating that ^{13}C - ^1H dipole-dipole interactions dominate the ^{13}C relaxation.

The relaxation behaviour of the peaks at 63.8 and 73.2 p.p.m. could not be interpreted quantitatively because carbons of both glycerol and erythritol contributed to each resonance. In order to interpret the data for the mannitol resonances, similar measurements were made on a 0.45 M solution of mannitol in D_2O , this concentration being chosen on the assumption that

Fig. 1. Natural-abundance ^{13}C NMR spectra of intact *Penicillium janczewskii* mycelia grown in shake-flask culture in media containing NaCl. Spectra were recorded at 25.05 MHz with 70° radio-frequency pulses applied at 2.0 s intervals, and processed with 1.55 Hz exponential broadening. (a) 0.99 a_w , 4000 scans; (b) 0.92 a_w , 2000 scans; (c) 0.85 a_w , 2000 scans. Intensities are not directly comparable because of variations in packing density (average 1.0 g ml^{-1}). These variations also affect linewidths, as shown by comparison of (a) and (b) with (c), which has a greater integrated intensity than (b). Peaks are labelled in (b) as follows: G, glycerol; M, mannitol. Erythritol also contributes to the glycerol resonances.

mannitol contributed approximately half to the intracellular concentration of osmotically active solutes (external concentration 0.9 M). T_1 values for C-1/C-6, C-2/C-5 and C-3/C-4 were 0.53, 0.86 and 0.83 s, respectively. All resonances had the full NOE. The T_1 values for the methine carbons were identical within the experimental error, suggesting that internal motions about the bonds connecting these four carbons were slow compared with the rate of overall molecular tumbling, and that the latter was not highly anisotropic. However, the methylene carbons had an average T_1 slightly longer than half that of the methine carbons, indicating that they experienced relatively fast internal motion. From the average T_1 for C-2 to C-5, a correlation time (τ_R) for overall molecular motion of 0.058 ns could be calculated using the equation for ^{13}C - ^1H dipolar relaxation in a rigid isotropically reorienting molecule (e.g. Norton *et al.*, 1982). For mannitol in the intact mycelia, τ_R was 0.11 ns, indicating that overall molecular motion within the cell, while still quite fast, was slower by a factor of 1.9 than that of 0.45 M-mannitol in aqueous solution. This effect could be due to a weak interaction of mannitol with one or more macromolecular components in the cell, or to a 1.9-fold higher viscosity of the cytoplasm relative to the aqueous solution.

In order to establish if the relaxation times of mannitol in aqueous solution were highly concentration-dependent, measurements were also made on a 0.9 M solution. T_1 values were 0.45, 0.69 and 0.74 s, respectively, corresponding to an overall rotational correlation time of 0.069 ns. Thus, if the intracellular concentration of mannitol were 0.9 M, then the difference between the rate of overall molecular motion in the cell and that in aqueous solution would be 1.6-fold, instead of 1.9-fold.

Fig. 2. Natural-abundance ^{13}C NMR spectra of aqueous ethanol extracts of *Penicillium digitatum* grown in media of 0.94–0.92 a_w . Spectra were recorded at 25.05 MHz with 200 scans, 60° radio-frequency pulses applied at 10.0 s intervals, and processed with 1.1 Hz exponential broadening. (a) NaCl, 0.94 a_w ; (b) sorbitol, 0.92 a_w ; (c) glucose-fructose, 0.92 a_w . Peak labels are: G, glycerol; M, mannitol; A, arabinoside; E, erythritol; S, sorbitol.

Mycelial extracts

Although the resonances arising from the polyols and other solutes in intact mycelia were fairly well resolved, the resolution at the magnetic field strength available was not sufficient to allow accurate quantification of the minor polyols. To overcome this, aqueous ethanol extracts of the fungi were studied. Figure 2 shows spectra of extracts of *P. digitatum* grown in three different media at 0.94–0.92 a_w . Peak resolution is considerably better than in spectra of intact mycelia due to a marked reduction in linewidths.

Figure 2(a) shows that glycerol was the dominant solute in *P. digitatum* grown on NaCl medium at 0.94 a_w , although a low level of mannitol was present. Resonances from sorbitol

Table 1. ^{13}C chemical shifts of fungal solutes

The chemical shifts were measured as described under Methods, at concentrations of 50 mg ml⁻¹ in D₂O at 25 °C.

Solute	C-1	C-2	C-3	C-4	C-5	C-6
Glycerol	63.82	73.22	63.82			
Erythritol	63.86	73.14	73.14	63.86		
Arabitol*	64.17†	71.50	71.70	72.17	64.25†	
Mannitol*	64.45	72.01	70.45	70.45	72.01	64.45
Sorbitol*	63.67†	72.21‡	70.88	72.32‡	74.19	64.06†
α -D-Glucopyranose§	93.30	72.67	74.00	70.84	72.67	61.80
β -D-Glucopyranose§	97.12	75.36	77.00†	70.84	77.16†	61.95
β -D-Fructopyranose§	64.56	99.27	70.92	68.78	70.41	65.11
β -D-Fructofuranose§	63.86	102.74	75.68	76.57	81.91	63.63
Trehalose§	94.43	73.38	73.77	70.92	72.28	61.78

* Assignments taken from Voelter *et al.* (1970).

† Assignments may be interchanged within the same row.

‡ Assignments may be interchanged.

§ Assignments taken from Rosenthal & Fendler (1976).

Fig. 3. Effect of water activity on the internal solutes of five fungi grown on sorbitol medium. (a) *Penicillium digitatum*; (b) *P. janczewskii*; (c) *Eurotium chevalieri*; (d) *Xeromyces bisporus*; (e) *Wallemia sebi*. □, sorbitol; ●, glycerol; ○, erythritol; ■, arabitol; ▲, mannitol; ▼, trehalose.

dominated the spectrum of the fungus grown on sorbitol medium at 0.92 a_w (Fig. 2b), although resonances from glycerol, erythritol, arabitol and mannitol were also observed. Similarly, resonances from glucose and fructose were prominent in the spectrum of *P. digitatum* grown on glucose/fructose medium at 0.92 a_w , but in this case glycerol was the major solute, and resonances were observed from arabitol and mannitol (Fig. 2c). Thus, Fig. 2 illustrates that one or more resolved resonances could be observed for the relevant polyols, even in the presence of resonances from sorbitol or glucose and fructose. The ^{13}C chemical shifts of all the relevant solutes, measured under the same conditions as employed for the extracts, are given in Table 1.

The responses of all five species of fungi to changes in a_w on sorbitol media are shown in Fig. 3. The non-xerophilic species, *P. digitatum* (Fig. 3a), exhibited the most complex solute pattern. Concentrations of both glycerol and erythritol rose as a_w fell. The amount of arabitol detected rose slightly, and the concentrations of mannitol and trehalose fell. In *P. janczewskii* (Fig. 3b),

Table 2. Effect of glucose/fructose on the concentrations [$\text{mmol (g wet wt)}^{-1}$] of internal solutes of five fungi

Internal solute	a_w :	<i>Penicillium digitatum</i>			<i>P. janczewskii</i>					
		0.99	0.96	0.92	0.99	0.90	0.83			
Glucose		5.1	20	32	11	188	238			
Fructose		3.5	14	34	12	162	202			
Glycerol		5.2	30	64	15	82	125			
Erythritol		4.0	6.5	—	5.7	21	14			
Arabitol		2.0	6.0	14	3.2	—	—			
Mannitol		6.3	8.8	7.3	17	—	10			
Trehalose		0.7	—	—	—	—	—			
Internal solute	a_w :	<i>Eurotium chevalieri</i>			<i>Xeromyces bisporus</i>			<i>Wallemia sebi</i>		
		0.99	0.88	0.78	0.96	0.88	0.75	0.99	0.88	0.83
Glucose		4.1	45	275	29	71	124	22	166	316
Fructose		4.2	40	218	18	51	88	21	128	238
Glycerol		tr	87	300	50	98	134	19	107	215
Erythritol		tr	6.8	—	22	19	—	—	—	—
Arabitol		tr	—	—	—	—	—	18	43	39
Mannitol		6.7	18	10	—	—	—	9.4	13	15
Trehalose		2.1	—	10.7	—	—	—	3.2	—	—

Tr, Not quantified because of peak overlap. Concentrations less than 1 mmol (g wet wt) $^{-1}$.

the concentration of glycerol rose steadily as a_w was lowered, there was a slight rise in erythritol, and the amount of mannitol remained steady. No arabitol or trehalose was detected. The solute pattern for *E. chevalieri* (Fig. 3c) was similar to that of *P. janczewskii* (Fig. 3b) except that erythritol was not detected. In the *X. bisporus* extracts (Fig. 3d), only sorbitol and glycerol were detected, except at the highest a_w . In extracts from *W. sebi* (Fig. 3e), there was a dramatic rise in the concentration of sorbitol in response to lowered a_w , and there was also a rise in glycerol concentration. Small amounts of arabitol and mannitol were detected in the extracts, at high and medium a_w , but not at the lowest a_w value.

The patterns of solutes detected in extracts from the fungi when grown on glucose-fructose were qualitatively similar to those obtained from growth on sorbitol media (Table 2). All fungal extracts contained large amounts of the external solutes. The most complex pattern of solute accumulation was again that of *P. digitatum* and that of *X. bisporus* was the simplest. *Penicillium janczewskii* and *E. chevalieri* responded similarly to increasing concentrations of external glucose/fructose.

When NaCl was used as the controlling solute, the patterns of internal solutes were quite different (Fig. 4). The lowest a_w at which *P. digitatum* (Fig. 4a) grew in NaCl was 0.94 (compared with 0.92 in the organic solutes). Glycerol was produced in increasing amounts as the a_w was reduced. No erythritol or arabitol was detected, and the other compounds that were detected at high a_w , mannitol and glucose, were not present at the lowest a_w . In the presence of NaCl, both *P. janczewskii* (Fig. 4b) and *E. chevalieri* (Fig. 4c) accumulated high levels of glycerol at intermediate a_w , but little at high or low values. However, the accumulation patterns for the other solutes were quite different for these two species. In *P. janczewskii*, the level of erythritol increased as the a_w was reduced, mannitol concentrations remained steady, and the level of arabitol rose slightly. No erythritol or arabitol was detected in *E. chevalieri*, and the mannitol which was present at the highest a_w was barely detectable at the lowest a_w . In extracts from *X. bisporus* (Fig. 4d), the level of glycerol decreased at reduced a_w . A small amount of glucose was seen at the highest a_w (0.96), but no other solutes were detected.

The relative amounts of the polyols in extracts of *P. janczewskii* grown on NaCl media were somewhat different from those observed in intact mycelia. At 0.99 a_w , concentrations of glycerol and erythritol in intact mycelia were similar to that of mannitol, whereas in extracts, mannitol was lower. At 0.92 a_w , glycerol was the major solute observed in both extracts and intact mycelia,

Fig. 4. Effect of water activity on the internal solutes of five fungi grown on NaCl media containing 5% glucose. (a) *Penicillium digitatum*; (b) *P. janczewskii*; (c) *Eurotium chevalieri*; (d) *Xeromyces bisporus*; (e) *Wallemia sebi*. ●, glycerol; ○, erythritol; ■, arabitol; ▲, mannitol; △, glucose.

but mannitol was higher in the mycelia than in the extracts. At 0.85 a_w , glycerol was the major solute in intact mycelia, whereas in extracts a significant amount of erythritol was also present, as well as smaller quantities of mannitol and arabitol. These differences may reflect the greater age of the cultures from which extracts were prepared.

Wallemia sebi (Fig. 4e) produced steadily increasing amounts of glycerol as the a_w was reduced. Arabitol, glucose and mannitol were also detected in the extracts when *W. sebi* was grown on NaCl media at high and medium a_w . Unlike the other xerophilic fungi studied, *W. sebi* produced similar quantities of glycerol when grown at 0.80 a_w on sorbitol- and NaCl-based media.

DISCUSSION

Glycerol is accumulated by a variety of micro-organisms in response to osmotic stress, and those species that are able to synthesize and retain considerable concentrations of glycerol have most successfully adapted to low a_w environments. With the exception of the halophilic bacteria, which accumulate K^+ , micro-organisms which are able to grow at or below 0.85 a_w use glycerol to maintain their intracellular osmotic balance (Borowitzka, 1981).

Pitt (1975) defined a xerophilic fungus as one which 'is capable of growth, under at least one set of environmental conditions, at a water activity below 0.85'. A number of food spoilage fungi are capable of growth below 0.85 a_w , and this study demonstrates that glycerol plays an important role in enabling them to grow at such low a_w .

For an organic compound to be suitable as an internal solute under these conditions, it must fulfil two requirements. It must be a small, highly soluble molecule, and it must allow normal cellular functions to continue in its presence. Brown & Simpson (1972) first proposed the term 'compatible solute' to describe a solute which could be accumulated to high concentrations by a cell, while still allowing its enzymes to function effectively.

Many low molecular weight polyols and polyol derivatives fulfil these requirements. Glycerol, being the smallest of the polyols has the greatest potential for lowering intracellular a_w , is extremely soluble in water, and appears to be less inhibitory to enzymes than a number of other polyols and sugars (Simpson, 1976).

Although it has been known for some time that glycerol was accumulated by *Dunaliella* species (Craigie & MacLachlan, 1964; Ben-Amotz & Avron, 1973; Borowitzka & Brown, 1974) and by *Saccharomyces rouxii* (Brown, 1978), the first reports of glycerol accumulation by

filamentous fungi appeared only in 1980. Luard (1980) showed that when *Chrysosporium fastidium* was grown on media containing glucose as the controlling solute, both glycerol and glucose were accumulated intracellularly as a_w was reduced. The lowest a_w studied was 0.865. *Chrysosporium fastidium* is an extremely xerophilic fungus that is capable of growth down to 0.69 a_w under favourable conditions (Pitt & Christian, 1968). Although it grows strongly on media containing glucose and/or fructose, it is highly intolerant of other solutes, growing slowly on media containing glycerol, and poorly or not at all when NaCl is used as the controlling solute (Pitt & Hocking, 1977). Luard (1980) was unable to grow *C. fastidium* on media containing KCl as the controlling solute. The fungus investigated in this study that most closely parallels this behaviour is *Xeromyces bisporus*, which occupies a similar ecological niche to *C. fastidium* (Pitt, 1975). This study showed that *X. bisporus* was able to accumulate glycerol at low a_w if either glucose/fructose or sorbitol was used to control a_w , but not if NaCl was the controlling solute.

Luard (1980) also demonstrated that *Penicillium chrysogenum* accumulated glycerol in response to decreasing a_w , when either glucose or KCl was used as the controlling solute. *Penicillium janczewskii*, investigated in this study, has very similar water relations to *P. chrysogenum* (Hocking & Pitt, 1979). These species have similar growth rates at equivalent a_w values, and are capable of growth down to at least 0.78 a_w . Both species accumulated glycerol when glucose and/or fructose were used to control a_w . *Penicillium janczewskii* also accumulated glycerol when grown on sorbitol-based media. When NaCl or KCl was used to control a_w , both species accumulated glycerol down to approximately 0.93 a_w . This was the lowest a_w at which *P. chrysogenum* was studied by Luard (1980). Our results show that, when *P. janczewskii* was grown on a NaCl-based medium at 0.85 a_w , the internal glycerol concentration was significantly lower than at 0.92 a_w . A similar effect was observed for *Eurotium chevalieri*, which is only slightly more xerophilic than *P. janczewskii*.

At the lowest a_w at which these two species were grown in NaCl (0.85), several weeks incubation was necessary to produce sufficient mycelium for analysis. It is possible that a significant proportion of the hyphae was already dead, and any accumulated internal solute had leaked into the growth medium. If only a small proportion of the mycelium had been viable at the time of extraction, then the value obtained for the concentration of intracellular glycerol would be erroneously low. Alternatively, it is possible that these species use some other osmoregulator, such as K^+ , when grown at low a_w on NaCl media. This area requires further investigation.

Overall, *P. janczewskii* and *E. chevalieri* exhibited similar responses to the various controlling solutes. One significant difference was the consistent production by *P. janczewskii* of erythritol. This polyol could also act as a compatible solute, and may be more easily retained by fungal membranes than glycerol.

The non-xerophilic species, *Penicillium digitatum*, accumulated considerable quantities of glycerol, even on NaCl-based media, over its relatively limited a_w range for growth. This was surprising in view of the fact that even under the most favourable conditions, it will not grow below 0.90 a_w (Hocking & Pitt, 1979). It may be that *P. digitatum* is able to produce glycerol, but below 0.90 a_w is unable to retain it intracellularly against a strong concentration gradient, a similar effect to that reported by Brown (1978) for *Saccharomyces cerevisiae*.

Wallemia sebi was the only xerophilic species which was able to retain increasing concentrations of glycerol when grown on NaCl-based media. *Wallemia sebi* is one of a small number of micro-organisms adapted to grow in high concentrations of salt. However, unlike some halophilic bacteria and algae, *W. sebi* does not have a physiological requirement for NaCl, but a preference for reduced a_w (Pitt & Hocking, 1977).

The results presented here demonstrate that natural-abundance ^{13}C NMR spectroscopy is a useful technique for studying internal solutes in both intact fungi and in aqueous extracts. It has been used previously to examine changes in ageing mycelia of a non-xerophilic fungus, *Penicillium ochrochloron* (Matsunaga *et al.*, 1980, 1981). In the study presented here, spectra of intact fungi exhibited broader resonances than those of extracts, due mainly to the heterogeneity of the samples, but the major solutes were still readily observed and identified. This indicated that these solutes were fairly mobile. In the case of mannitol in intact *P. janczewskii* mycelia,

NMR relaxation measurements showed that its overall rotational motion was about 1.9-fold slower than in aqueous solution, suggesting a higher internal viscosity in the mycelia. In intact cells of marine and salt-tolerant algae, the rotational mobilities of the osmoregulatory solutes were about half those of corresponding aqueous solutions, indicative of internal viscosities up to twice those of the aqueous solutions (Norton *et al.*, 1982).

At higher magnetic field strengths, resolution and sensitivity should be sufficient to permit the observation of even minor solutes in intact mycelia. Furthermore, relaxation measurements on the major solutes at two different field strengths may be expected to yield a more detailed picture of the motional behaviour of these solutes in intact mycelia than that which emerged from data at a single field strength.

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