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Yeast water stress physiology

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<tr>
<td>$a_w$</td>
<td>Water activity</td>
</tr>
<tr>
<td>BYM</td>
<td>Basal yeast medium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3' 5'-monophosphate</td>
</tr>
<tr>
<td>c.f.u.</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>log$_{10}$</td>
<td>Logarithm to base 10</td>
</tr>
<tr>
<td>MA</td>
<td>Malt extract agar</td>
</tr>
<tr>
<td>SHA</td>
<td>Synthetic honey agar</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NAD$^+$</td>
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<td>NADP$^+$</td>
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ABSTRACT

When growing exponentially in batch culture *Saccharomyces cerevisiae* passed through a phase in which about one cell in $10^4$ survived a water stress imposed by plating onto low water activity ($a_w$) agar. This phenomenon was called water stress plating hypersensitivity. Stationary phase cultures were resistant to a water stress of the same magnitude. Most of the other yeast species tested did not display plating hypersensitivity, with the exception of *Candida krusei* and a *Kloeckera* isolate.

The difference between viable count on high $a_w$ agar and low $a_w$ agar (the difference between the log values being called the plating discrepancy) was diminished in exponentially growing *S. cerevisiae* if the yeast was incubated in a glucose solution of intermediate $a_w$ before being plated onto the stressing agar. Addition of glycine betaine and glycerol to the stressing agar, two substances known to improve the survival of some microorganisms at low $a_w$, did not improve the viability of *S. cerevisiae*. The proportion of the population able to grow on the stressing agar was dependent on the agar $a_w$. If the agar contained 5% NaCl (mass/vol.) or less, the counts on salt and high $a_w$ agars were the same. The duration of the plating hypersensitivity period during the growth cycle of *S. cerevisiae* could be prolonged by incubating the culture at 15°C rather than 30°C and by increasing the glucose content of the culture medium. Growth under anaerobic conditions also prolonged the duration of the hypersensitivity period.

The nature of the carbon source in liquid culture did not influence greatly the response of *S. cerevisiae* to a water stress. When ethanol or maltose instead of glucose was the sole carbon and energy source, *S. cerevisiae* still displayed water stress plating hypersensitivity.
The magnitude of the plating discrepancy showed a correlation with growth rate when the rate was limited by nutrient flux but not when it was affected by a change in temperature. Continuous culture studies with glucose as limiting substrate demonstrated that *S. cerevisiae* was more resistant to a water stress when growing slowly.

Those species resistant to a water stress during the exponential growth phase contained significant quantities of glycerol and at least one other polyol (erythritol, arabitol and mannitol), as determined by thin-layer chromatography. In hypersensitive yeasts, such as *S. cerevisiae* none or only trace amounts of glycerol were detectable. It was concluded that polyols were protecting the resistant yeasts against the effects of a sudden water stress and that water stress plating hypersensitivity was largely confined to nonxerotolerant yeasts.

The stationary phase resistance of *S. cerevisiae* was attributed to the disaccharide trehalose. The glycerol content remained low in the stationary growth phase, unlike the trehalose content which was approximately 5% of the dry mass in stationary phase cultures. The role of trehalose in preventing plating hypersensitivity was evidenced by the correlation between the magnitude of the plating discrepancy and trehalose content under a variety of experimental conditions, the persistence of water stress plating hypersensitivity in the stationary growth phase of a *glc1* mutant defective in trehalose accumulation and the large plating discrepancy in stationary phase cultures treated with 2,4-dinitrophenol, a substance which induces the breakdown of trehalose.

It was concluded that trehalose, like the polyols in the resistant species, was protecting *S. cerevisiae* against the effects of a sudden water stress.
INTRODUCTION

Nearly all yeasts tolerate rather than require dry conditions for growth (section 1.2.1.2). Xerotolerant yeasts tolerate extremely dry conditions by modifying their intracellular environment with glycerol, a solute which is "compatible" with enzyme function even at high concentrations (Brown and Simpson 1972). Over a wide range of water activities the content of glycerol is controlled by yeasts so that approximately constant turgor pressure and/or volume is maintained. This process is known as osmoregulation (section 1.1.3).

*S. cerevisiae* is a nonxerotolerant yeast, yet it too osmoregulates with glycerol. The mechanism it uses to adjust its glycerol content is different from and less effective than that of xerotolerant yeasts such as *S. rouxii* (section 1.2.2.2). *S. cerevisiae* and *S. rouxii* also differ in their processes of adaptation to a sudden decrease in water activity (Edgley and Brown 1983). The adaptation of *S. rouxii* to low water activities is apparently simple whereas the adaptation of *S. cerevisiae* is relatively complex. Edgley and Brown (1983) describe two stages in the adaptation process of *S. cerevisiae*. *S. cerevisiae* shows a catastrophic drop in apparent viability in the first 50 hours (stage 1) after being transferred to growth medium containing 10% sodium chloride. This is followed by a gradual recovery of viability (stage 2) to the original levels over the next 100 hours. Glycerol is synthesized by both yeasts immediately after transfer to low water activity medium. However an increase in glycerol-3-phosphate dehydrogenase activity is not observed until the beginning of stage 2 in *S. cerevisiae*.

The major emphasis of this study was to investigate factors influencing the survival of yeasts when subjected to a sudden water stress. In particular experiments were conducted to characterize further the complex viability changes associated with the adaptation of *S. cerevisiae* to media containing high concentrations of solutes.
CHAPTER ONE: LITERATURE SURVEY
1.1 WATER STRESS

Water is essential for the growth and reproduction of microorganisms. Its physiological functions have been summarized by Pirt (1975). If the water availability in an environment changes sufficiently to retard growth of the microorganism and its ability to reproduce, the microorganism may be said to be subject to a water or osmotic stress (Rose 1976). Either an increase or decrease in environmental water availability may impose a water stress on a microorganism. As research has concentrated on water stresses caused by a deficiency of water this discussion will be largely confined to this type of water stress.

1.1.1 Water availability

The status of water in microbial cells and the substrates on which they grow are normally described in thermodynamic terms, water activity ($a_w$) osmotic pressure ($\pi$) and water potential ($\Psi$) being most widely used. Several recent reviews (Brown 1976; Griffin 1981; Wyn Jones and Gorham 1983) have detailed the thermodynamic derivations of these terms in order to illustrate their relation to one another and their applicability to studying microbial water stress.

Water potential, which is the partial molal free energy of water, can be resolved into several components:

$$\Psi = \psi_s + \psi_m + \psi_p$$  \hspace{1cm} (1)

where $\Psi$ is the total water potential and $\psi_s$, $\psi_m$ and $\psi_p$ are the solute, matric and pressure components, respectively. The component $\psi_s$, sometimes ambiguously called 'solute potential' but better known as osmotic potential is equivalent but opposite in sign to osmotic pressure ($\pi$) and represents the influence of dissolved substances on water. The pressure potential, $\psi_p$, represents the hydrostatic pressure ($P$) component of $\Psi$, such as the positive $\psi_p$ identified with the turgor pressure of cells. Matric potential, $\psi_m$, represents the variation in $\Psi$ due to
liquid-gas and liquid-solid interactions. Nobel (1974) and Passioura (1980) have emphasized that interface effects on water availability can be incorporated in $P$ and $\pi$ although when interfacial forces are significant it is convenient to express these forces separately as $\psi_m$.

Water potential is useful when considering the water availability in systems with significant solid components such as soil, timber etc. (Griffin 1981). The water available in such systems is significantly reduced because of matric forces, the term in equation 1, $\psi_m$ becoming increasingly important as the water content of the system decreases. Dry environments, those environments with low water availability, have been divided by Griffin (1981) into those where $\psi_m$ contributes to the low water availability (soil, stored food) and those where it does not (salt lakes, sugary syrups, brines). The water relations of microorganisms that predominate in environments with a significant $\psi_m$, such as filamentous fungi, are often described in terms of water potential.

The status of water inside microbial cells is a function of the three components given in equation 1. At equilibrium:

$$\psi^e = \psi^c = \psi_s^c + \psi_m^c + \psi_p^c$$  \hspace{1cm} (2)

where the superscripts $e$ and $c$ indicate environment and cell, respectively. The cell $\psi_m^c$ component denotes intracellular matric potential. In organisms growing under optimal conditions $\psi_m^c$ is considered to be unimportant (Adebayo et al. 1971; Griffin 1978) but it may contribute significantly to $\psi^c$ when a microorganism is subjected to a severe decrease in environmental $\psi$ (Schobert 1977). The summation of $\psi_s^c$ and $\psi_m^c$ components is equal to negative osmotic pressure ($\pi$). An intracellular hydrostatic or turgor pressure is generated ($P$, equivalent to the $\psi_p^c$ component in equation 2) so that, at equilibrium, $\psi^e$ equals $\psi^c$ (equation 2) and

$$\psi^c = P - \pi$$  \hspace{1cm} (3)
Since Scott (1957) reviewed the water relations of food spoilage microorganisms in terms of water activity \((a_w)\), this term has been widely used when the effects of aqueous solutions on microorganisms are being considered. Thus the water relations of those microorganisms whose natural environments are solutions (such as most yeasts, bacteria and algae) are most often described using water activity. Water activity is directly related to \(\Psi\) by its natural logarithm although the \(\psi_m\) and \(\psi_p\) components in equation 1 are essentially negligible in solutions. The water activity may be defined by the equation:

\[
aw = \frac{p}{p_0} = \frac{n_2}{n_1 + n_2}
\]

where \(p\) and \(p_0\) are the vapour pressures of the solution and solvent (pure water), respectively and \(n_1\) and \(n_2\) are the number of moles of solute and solvent, respectively. Detailed treatments of the concept of water activity are found in reviews by Scott (1957), Kushner (1971), Brown (1976) and Griffin (1981). Among the advantages of using \(a_w\) instead of \(\Psi\) when studying solution effects on microorganisms is the ease with which water activity can be determined (Brown 1978a): water activity is equal to the equilibrium relative humidity of a solution (expressed as a percentage) divided by 100. Water activity is also a convenient means of expressing the water status of microbial substrates such as stored food products where only the totality of the water status is significant (Griffin 1981).

1.1.2 Microbial growth

In 1957 Scott made several generalizations about the growth response of microorganisms to a water stress (decrease in environmental water activity): as the environmental water activity is reduced below an optimum level there is an increase in the lag period and a decrease in growth rate and cell mass synthesized; all microorganisms are likely to have a characteristic optimum environmental water activity at which growth occurs most rapidly; the growth response of many microorganisms to a particular water activity is independent of the type of solute
used to adjust it. Much information concerning these generalizations have been published since 1957 for numerous microorganisms and their validity will be discussed, with specific reference to yeast, in section 1.2.

Data published on the growth responses of a wide variety of microorganisms has enabled microorganisms to be grouped on the basis of their growth response to environmental water activity (Kushner 1978; Harris 1981; Griffin 1981; Larsen 1986). Two broad groups of microorganisms are recognized, those that tolerate low water activities but grow best at high water activities (xerotolerant species) and those that fail to grow at high water activities (xerophilic species). The prefix halo- is usually employed instead of xero- when the tolerance of or requirement for low water activity is dependent on salts being used to adjust the water activity. Xerotolerant organisms can be further subdivided into slightly, moderately and extremely xerotolerant groups. Similarly, xerophiles can be divided into slightly, moderately and extremely xerophilic species.

Griffin (1981) has emphasized that the division of microorganisms on the basis of their growth response to environmental water activity is somewhat arbitrary in the sense that each group merges into the next. In addition, environmental factors such as temperature, pH and nutrition have marked influences on growth optima and the range of water activities at which a microorganism will grow (Brown 1976, Griffin 1981).
1.1.3 Osmoregulation

In the absence of an explicit water transport apparatus in the cell plasma membrane (Kotyk 1973), microorganisms can absorb water only by lowering their cellular water potential below that of the environment (Brown 1979, Griffin 1981). Turgor pressure is generated to balance the lower osmotic potential and the pressure so developed is the driving force for cell growth (see Cosgrove 1986).

Brown (1976, 1979) has described the response of a microorganism to a change in environmental water activity. Three phases are recognized. In phase 1 the microorganism rapidly adjusts thermodynamically to the new environment water activity, this process involving a water flux into (in the case of increased environmental water activity or upshock) or out of (decreased environmental water activity or downshock) the cell until equilibrium is restored. The water flux may be accompanied by cell shrinkage (downshock) or swelling (upshock) in microorganisms without cell walls, as seen in the algae _Dunaliella salina_ (Trezzi et al. 1965) and _Poterioochromonas malhamensis_ (Kauss 1977). In walled cells changes in turgor pressure rather than volume may occur during the first phase.

In the second phase (Brown 1976, 1979) the microorganism, if it has survived the first phase, recovers its original volume (wall-less microbial cells) or turgor (walled cells). In some situations complete recovery of volume or turgor pressure to original, pre-stress levels does not occur yet such organisms are able to enter phase 3, which is characterized by the resumption of growth. Incomplete recovery of original turgor pressure following a change in environmental water activity has been demonstrated in several macroalgae (Kirst and Bisson 1979; Reed et al. 1980; Dickson et al. 1980) although this is not characteristic of all macroalgae (see for example Bisson and Gutknecht 1977). Similarly, incomplete cell volume recovery has been reported in _Teraselmis subcordiformis_ (Kirst 1977), a wall-
less alga, while another genus of wall-less alga, \textit{Poterioochromonas} does recover original volume after a change in environmental water activity (Kauss 1977).

Recovery of original turgor and volume, at least to the point where growth is possible, is accomplished in phase 2 either by absorbing water or dispelling water. Such changes in cellular water content are achieved by adjusting solute content, involving metabolic conversion within the cell or solute transport, or both. As the solute content changes, water fluxes maintain the thermodynamic parity between cellular and environmental water potential. A microorganism has reached phase 3 when growth resumes at the new environmental water activity. It is fully adapted to the new conditions in phase 3 and is phenotypically different from its condition in the previous environment (Brown 1976, 1979).

The term osmoregulation has been used to describe the response of a cell to a new environmental water activity, whereby approximately constant cell volume and turgor pressure are maintained in the face of a changing environmental water activity by appropriate adjustment of intracellular solute content (Brown and Edgley 1980). However the suitability of using the term osmoregulation to describe the maintenance of approximately constant turgor and/or cell volume has been questioned by Cram (1976) and Reed (1984). Turgor regulation (in the case of walled cells) and volume regulation (wall-less cells) have been suggested as being more informative terms although the interrelation between volume and turgor (Zimmerman 1978; Bisson and Gutknecht 1980) casts doubt on whether a distinction between turgor and volume regulation can be made. Other terms have been put forward by Reed (1984) which he suggests are more suitable, if used appropriately, in describing the response of a microorganism to a water stress.
1.1.4 Compatible solutes

The principal solutes, known as osmoregulatory solutes, accumulated in response to a decrease in environmental water activity in a variety of microorganisms have been tabulated by Borowitzka (1981) and Yancey et al. (1982). Four classes of osmoregulatory solutes have been found in microorganisms, these being polyhydric alcohols, carbohydrate derivatives, $K^+$ (KCl) and free amino acids and their derivatives. In xerotolerant and xerophilic microorganisms growing at extremely low water activities the cellular concentration of these solutes is very high. For example, in *Halobacterium salinarium* growing in 4.0M salt, the internal concentration of $K^+$ is 4.6 molal (Christian and Waltho 1962) and similar or higher levels of $K^+$ have been reported in other halophilic bacteria. The only other osmoregulatory solute known under extreme conditions is glycerol, present in molar concentrations in halophilic species of *Dunaliella* and xerotolerant yeast (Brown 1976, 1978).

In 1976 Brown proposed two mechanisms to explain how a microorganism is able to grow with high solute concentrations inside and outside the cell. An organism utilizing mechanism 1 has characteristically different proteins from those of other organisms, which enables it to function inherently better at low water activities. Those microorganisms utilizing the second mechanism, mechanism 2, modify their intracellular conditions so that the detrimental effect of of low water activity on enzyme activity, and cell function in general, is diminished. If distinctive metabolic pathways are utilized by a microorganism at low water activities, further division is possible into those that contain enzymes which function intrinsically better at low water activities (mechanism 1A) or those which produce an end product which modifies the intracellular environment so as to diminish the environmental inhibition caused by low water activities (mechanism 2A).
The salt requirement of halophilic bacteria can be largely explained in terms of mechanism 1 although the intracellular environment is also modified in these bacteria (mechanism 2). The physiological and biochemical peculiarities that determine the salt requirement of these bacteria have been reviewed by Brown (1983) and Kushner (1978, 1985, 1986).

All xerotolerant and xerophilic eucaryotes depend on mechanism 2. The osmoregulatory solutes accumulated by these microorganisms have been called "compatible solutes" (Brown and Simpson 1972, Aitken and Brown 1972) because at high concentrations they allow enzymes to function effectively. Many of the solutes that are able to function as osmoregulatory solutes under mild water stresses are not found as the major osmoregulatory solutes at lower water activities. Presumably this is because these solutes are insufficiently soluble and too toxic or inhibitory, thus incompatible with cell function, at the concentrations that would be required under severe water stresses. The distinctive properties of compatible solutes such as high solubility, minimal alteration of enzyme structure and minimal inhibition of enzyme function has been discussed in detail by Brown (1976, 1978, 1979) and Borowitzka (1981).
1.2 RESPONSE OF YEAST TO A WATER STRESS

Numerous yeast species have been isolated from natural and man-made low water activity environments. Species of *Candida, Debaryomyces, Hansenula, Pichia, Saccharomyces* and *Torulopsis* predominate in these environments. Details about their distribution can be found in reviews by Mrak and Phaff (1948), Scott (1957), Ingram (1957), Onishi (1963), Spencer (1968), Pitt (1975) and Tilbury (1980).

The nomenclature used to describe yeasts isolated from low water potential habitats is varied and includes osmophilic (Christian 1963, Scarr and Rose 1966), osmotophilic (Van der Walt 1970), osmotoduric (Van der Walt 1970), osmotolerant (Anand and Brown 1968) and osmotrophic (Sand 1973). Brown (1976) has preferred to use "xerotolerant" to describe yeasts able to grow at low water activities. Those yeasts requiring low water activities are called xerophilic (Pitt 1975, Brown 1976).

1.2.1 Growth

A water stress influences several aspects of yeast growth and multiplication. Scott's (1957) generalizations about the changes in the lag phase, growth rate and cell mass that occur in microorganisms when exposed to a water stress (decrease in environmental water activity) apply also to yeast. Several reports document a lengthening lag phase in liquid cultures (i.e. the incubation period between transfer of yeast to a lower water activity and detectable initiation of cell growth) with decreasing water activity (Phaff et al. 1952; Ross and Morris 1962; Norkrans 1966). Similarly, the lag phase lengthens with decreasing water activity when yeast are grown as semisolid cultures (Wei et al. 1982) or on agar (Horner and Anagnostopoulos 1973). Reduction in exponential growth rate (Norkrans 1966; Combs et al. 1968; Anand and Brown 1968; Watson 1970) and biomass yield i.e.
the total mass of yeast per unit volume of growth medium (Ross and Morris 1962; Norkrans 1966; Umemoto et al. 1967; Combs et al. 1968; Tanner et al. 1981a; Wei et al. 1982) also accompany diminishing water activities.

Changes in yeast viability accompanying a water stress have been reported by Onishi (1957a), Edgley and Brown (1983), Onishi and Shiromaru (1984) and Morris et al. (1986). During the lag phase that follows a water stress, catastrophic drops in plate counts occur, with the magnitude of the drop being dependent on the species, the severity of the stress, the water activity of the plating agar and the nature of the solute used to adjust the water activity of the stressing medium and the plating agar.

1.2.1.1 Solute effects

One of the other generalizations Scott (1957) made about the water relations of microorganisms is that the growth response of many microorganisms to a particular water activity is largely independent of the type of solute used to adjust the water activity. This does not appear to be the case in many yeasts species. Brown (1976), in recognition of the importance of solute type on the water relations of yeasts, subdivided xerotolerant yeasts into sugar-tolerant and salt-tolerant. This subdivision seems justified on the basis of growth studies by Onishi (1963) Rodriguez-Navarro (1971) and Anand and Brown (1968). Differences in the xerotolerance is not confined to growth comparisons between sugar and salt solutions. For example, Anand (1969) observed that a sugar-tolerant strain unable to grow at $0.85a_w$ in medium adjusted with sucrose could grow in the same medium when the medium water activity was lowered even further ($0.80a_w$) by addition of glycerol.

It is not uncommon for strains of the same species to have different tolerances of reduced water activity, depending on the solute used to adjust it (Onishi 1957b;
Anand and Brown 1968; Mori and Windisch 1982). This phenomenon is typified by *Saccharomyces cerevisiae* where differences in growth of five strains at various water activities adjusted with polyethylene glycol or sucrose have been reported by Anand and Brown (1968). Four of the five strains could not grow at 0.895$\text{aw}$ adjusted with sucrose but, at a slightly higher value (0.917$\text{aw}$), all but one strain (Y41) did grow, this strain grew at 0.935$\text{aw}$ and above. When polyethylene glycol was used to adjust the water activity, none of the strains could grow below 0.93$\text{aw}$ and one of these (Y43) was unable to grow below 0.95$\text{aw}$.

The difference in strain solute tolerance often reflects the environment from which the strains are isolated. For instance, sugar-tolerant *S. rouxii* strains isolated from marzipan could not grow in medium containing 3.5M NaCl, while most of the strains isolated from shoyu mashes and miso pastes were tolerant of more than 3.5M NaCl (Mori and Windisch 1982). Genotypic differences between salt sensitive and salt tolerant *S. rouxii* strains was alluded to by Mori and Windisch (1982). They illustrated that hybrids constructed between salt tolerant and salt-sensitive strains have the same phenotype as the salt-tolerant strains and concluded that salt tolerance is dominant over the salt sensitive character.

1.2.1.2 Distinction between xerotolerant and xerophilic yeasts
Because the water relations of yeasts vary with the solute used to adjust the water activity, it is difficult to propose a precise definition of xerotolerance or xerophilism (Brown 1976). There seems to be a reasonable consensus that yeasts capable of growing below 0.85 $\text{aw}$ are "osmophilic" (Christian 1963; Van der Walt, 1970) although 0.87 (Mossel 1971) and 0.865 (Scarr and Rose 1966) are considered by others to be the critical water activities. All these values have been specified in terms of sugar concentrations and only Van der Walt (1970) made a distinction between "osmotolerant" and "osmophilic" yeast: the former can grow on 0.90$\text{aw}$ glucose agar but not on 0.85$\text{aw}$ agar while the latter grow on both agars.
Pitt (1975) has proposed a broader definition of osmophilic yeasts, specifying that these yeasts are able to grow at water activities below $0.85a_w$ under at least one set of environment conditions. However, most yeasts that would be defined as osmophilic by this or any of the other definitions are, according to Anand and Brown (1968), sugar tolerant or xerotolerant (Brown 1976).

Only a few reports have documented yeasts with a requirement rather than a tolerance of low water activity. Xerophilic yeast should by definition (Ingram 1957; Walker 1977) require low water activities for growth. Griffin (1981) defined as xerophilic those microorganisms that fail to grow above $0.97 \ a_w$ and have minima at $0.70 \ a_w$ or less. Anand and Brown (1968) described a xerophilic yeast, a *Zygosaccharomyces nectarophilus* isolate, that cannot grow above $0.98 \ a_w$. It had a growth optimum at approximately $0.96 \ a_w$ but its very low growth rate even at this water activity probably indicates that the growth medium used in this study was not ideal for *Z. nectarophilus*. This yeast will grow at higher water activities in the same medium if the incubation temperature is reduced from 30°C to between 16 and 23°C (M Edgley, cited in Brown 1976). Similarly, *Torulopsis halonitratophila*, unable to grow at higher water activities at 30°C, will do so at 20°C (Onishi 1960b). Several other examples of temperature dependent xerophilic yeast have been described by Onishi (1963).

1.2.1.3 Improved tolerance

With the exception of the temperature-dependent xerophiles, yeasts able to grow at low water activities tolerate rather than require such conditions. Their xerotolerance can be reduced by prolonged cultivation in dilute media but tolerance can be restored by "training", i.e. transferring the yeast through a series of increasingly lower water activity solutions (Ingram 1950; Scarr 1951; English 1954; Onishi 1957a, 1963; Bellinger and Larher 1986).
Yeasts not known for their xerotolerance can also be trained to grow under conditions that they normally find totally inhibitory. For instance, if *Saccharomyces cerevisiae* is preadapted in 1.5M NaCl growth medium, it is able to grow at salt concentrations which are normally fully inhibitory (Bellinger and Larher 1986). The capacity for improving the tolerance of nonxerotolerant yeasts is not unlimited, however. Their tolerance of low water activities after training is still not as great as that of the xerotolerant species.

### 1.2.2 Intracellular composition

Exposure of a microorganism to low water potential results in a water efflux from the microorganism until equilibrium is restored (see sections 1.1.1 and 1.1.3). The efflux is accompanied by a shrinkage of the cell (Gibson 1973; Rose 1975; Corry 1976a, 1976b; Neidermeyer et al. 1977; Morris et al. 1986). The shrinkage is rapid and the shrunken yeasts appear 'phase-light' in the phase contrast light microscope (Morris et al. 1986); phase-light cells are normally regarded as non-proliferating (Bugeja et al. 1982). Major alterations in cell envelope ultrastructure accompany decreases in cell volume, at least in *Saccharomyces cerevisiae* (Kapecka et al. 1973; Neidermeyer et al. 1977; Morris et al. 1986).

In both xerotolerant and nonxerotolerant strains cell volume generally changes in response to water potential, regardless of the solute used to adjust the potential, although there are a few notable exceptions. The cell volumes of the nonxerotolerant strains studied by Rose (1975), *S. cerevisiae* and *Schizosaccharomyces pombe*, were virtually unaffected by polyethylene glycol (molecular weight 200) over a range of water activities. Apparently these yeasts equilibrated with the external water potential by admitting substantial amounts of polyethylene glycol into their cells.
In a shrunken state yeast cannot grow. As discussed in section 1.1.3 microorganisms regain their original volume and turgor and recommence growth by accumulating osmoregulatory solutes. Although glycerol is recognised as the major osmoregulatory solute in yeast, at least under a severe water stress, the contents of a few other solutes are known to increase with lowered water activity.

1.2.2.1 K⁺ and Na⁺

Norkrans and Kylin (1969) measured the K⁺ and Na⁺ content of the halotolerant Debaryomyces hansenii and the nonxerotolerant Saccharomyces cerevisiae during the first 20 hours of growth in medium of various NaCl concentrations. Both yeasts have the ability to regulate their internal salt compositions by increasing their K⁺ to Na⁺ ratios relative to the medium ratio. However the total salt level was not sufficient to account for the osmotic potential of the two yeasts and so Norkrans and Kylin concluded that the production of other solutes for the cell sap is involved in the response of these yeasts to low environmental water activity.

Under comparable conditions D. hansenii is more efficient than S. cerevisiae at extruding Na⁺ and taking up K⁺ from the medium (Norkrans and Kylin 1969; Hobot and Jennings 1981). High K⁺ concentrations are found in halophilic bacteria (Christian and Waltho 1962). The accumulation of K⁺ and the efficient extrusion of Na⁺ may be a major reason why D. hansenii is more salt tolerant than S. cerevisiae.

1.2.2.2 Glycerol

Since the discovery that all xerotolerant yeasts isolated from environments containing high sugar concentrations produce glycerol and often other polyols (including erythritol, arabitol and mannitol) in high yields when grown in 60% glucose medium (Spencer and Sallan 1956), low environmental water activity (i.e. high sugar or salt concentration) has been identified as the major environmental
factor that increases polyol production in these yeasts (Onishi 1963; Spencer 1968; Spencer and Spencer 1978). Analysis of the intracellular composition of xerotolerant yeasts revealed that a polyol(s) was the major trichloroacetic acid-soluble constituent at high water activities but no polyol was detectable in the nonxerotolerant yeasts (Brown and Simpson 1972; Brown 1974). When grown at low water activities glycerol was accumulated in proportion to the external water activity in the four yeasts that have been documented, the xerotolerant *Debaryomyces hansenii* (Gustafsson and Norkrans 1976; Adler and Gustafsson 1980), *Saccharomyces rouxii* (Brown 1978a; Edgley and Brown 1978) and *Hansenula* sp. (Ozawa and Iwamoto 1981) and the nonxerotolerant *S. cerevisiae* (Brown 1978a; Edgley and Brown 1978). The concentration of glycerol within these yeasts was dependent on the growth phase of the batch culture with highest levels being found in exponentially growing yeast. The time course of glycerol accumulation in *S. rouxii* (Edgley and Brown 1983; Onishi and Shiromaru 1984) and *S. cerevisiae* (Edgley and Brown 1983) during adaptation to high salinity medium has also been studied.

Another polyol, arabitol, is produced by xerotolerant yeasts (Onishi 1960a). It was found at a higher level than glycerol in stationary phase cultures of *D. hansenii* growing in 4mM and 2.7M NaCl media (Adler and Gustafsson 1980) and in *S. rouxii* growing in 0% and 5% NaCl, mass/vol., media (Brown 1978a). Intracellular arabitol level was not, however, enhanced by more than a factor of two in *D. hansenii* in low water activity medium and to a lesser extent in *S. rouxii*.

Brown (1978a) and Edgley and Brown (1978, 1983) demonstrated that the xerotolerant *S. rouxii* employs a different mechanism from the nonxerotolerant *S. cerevisiae* for attaining intracellular glycerol levels necessary for growth at low environmental water activities. *S. rouxii* retains an increasingly higher proportion of the glycerol it produces within the cell as water activity decreases. Its overall
glycerol production increases only marginally with decreasing water activities. In contrast, glycerol production in *S. cerevisiae* increases dramatically with lowered water activity, with most of the glycerol being found in the medium. Presumably the limit of water activity tolerance of *S. cerevisiae* is reached when an unacceptably high proportion of its total metabolic activity is diverted to glycerol production.

### 1.2.2.3 Nucleotides

A uridine nucleotide sugar, UDP-*N*-acetylglucosamine, is abundantly accumulated in *Saccharomyces rouxii* when grown on low water activity medium containing 2M glucose (Tomita 1983). It is not present in the intracellular pool when the yeast is grown in high water activity medium. The nucleotide is known to participate in the biosynthesis of chitin in some fungi. Whether UDP-*N*-acetylglucosamine metabolism is important in the osmoregulatory response of *S. rouxii* has not yet been established.

### 1.2.2.4 Amino acids

Amino acid accumulation as a response to lowered environmental water activity has been observed in many bacteria (Tempest et al. 1970; Measures 1975) and algae (reviewed by Borowitzka 1981). In yeast amino acids are reported to be the dominant group of soluble intermediates (Conway and Armstrong 1961).

Changes in the free L-lysine content of *Saccharomyces cerevisiae* growing in medium of various water activities have been reported by Tanner et al. (1981b). Intracellular free lysine concentration is at a maximum at the end of the exponential growth phase; inclusion of NaCl (0.15-0.6M) in the medium increases L-lysine concentrations at this stage of the fermentation. The highest concentration of L-lysine is found when grown in medium containing 0.6M NaCl and is about four times (relative to the mass of yeast) that level found during growth in medium
containing no extra salt. If cultivated anaerobically no such increase in L-lysine content is observed with increasing NaCl levels (Wei et al. 1982). The contribution of L-lysine to the osmoregulatory response of *S. cerevisiae* may be that of an osmoregulatory solute under mild water stresses.

In contrast to the findings of Tanner and colleagues, Adler and Gustafsson (1980) found no major change in the composition of the total amino acid pool of *Debaryomyces hansenii* in response to increased medium salinity, with the exception of a small rise in proline content. However it is interesting to note that at low salinity the total amino acid pool increases during the exponential growth phase and subsequently declines in stationary phase, a pattern observed for L-lysine in *S. cerevisiae*.

Adler and Gustafsson (1980) compared the amino acid content of *D. hansenii* in medium containing 4mm and 2.7M NaCl. It is possible that at an intermediate salinity one or more of the amino acids may have a higher content, the situation that is observed in *S. cerevisiae* (Tanner et al. 1981b). However, measurements of amino acid content at intermediate salinities in another xerotolerant yeast, *S. rouxii* (Brown and Stanley 1972) do not demonstrate a stimulation of amino acid content by decreased water activity.

### 1.2.2.5 Trehalose

One other metabolic product that increases with decreasing water activity is trehalose. Trehalose, α-D-glucopyranosyl α-D-glucopyranoside, is a nonreducing disaccharide of glucose. Its presence in microorganisms has been largely associated with its role as a storage carbohydrate, particularly in fungi. In the baker's yeast industry it is well known that a high trehalose content is desirable if the yeast is going to be dried (Bekers et al. 1981) or used in doughs with high sugar content (Edelmann et al. 1978).
Trehalose is often accumulated during periods of reduced growth rate in fungi, high concentrations of trehalose are common within spores and other reproductive and vegetative structures of fungi. Mobilization of trehalose typically occurs when these reproductive structures germinate. Thevelein (1984b) has extensively reviewed the conditions leading to trehalose accumulation and degradation in yeast as well as the current state of knowledge about trehalose regulation.

Edgley (1980) could only just detect trehalose in exponentially growing cells of *Saccharomyces cerevisiae* in high water activity medium. Although trehalose was not measured quantitatively by Edgley, an indication of the relative concentration of trehalose in cell extracts was gained by semiquantitative paper chromatography. Growth of *S. cerevisiae* at lower water activities in media supplemented with NaCl or polyethylene glycol (molecular weight 200) results in progressive increases in the trehalose content of fully adapted, exponentially growing cells. Trehalose is also present in *S. rouxii* but its intracellular concentration did not respond to the lowered water activity of the growth medium.

The trehalose content of *S. cerevisiae* increases during the adaptation period after transfer from a high water activity to a low water activity (10% NaCl) medium (Edgley and Brown 1983). Trehalose was first detected in *S. cerevisiae* 20 hours after transfer with increasing amounts of trehalose being found in subsequent samples (Margaret Edgley unpublished results). Trehalose is undetectable on paper chromatograms extracts of *S. rouxii* at any time during the adaptation process.

Increases in trehalose content in response to lowered water activity has also been reported for *Candida tropicalis* growing in a pH stat (Furyaeva et al. 1985). In
medium containing 110g/l of NaCl, trehalose content was twice that found in the absence of NaCl.

Ozawa and Iwamoto (1981) have reported a decline in trehalose content in *Hansenula* with increasing salt (KCl or NaCl) concentrations. At low salt concentrations trehalose is the major intracellular solute but at higher salt concentrations glycerol predominates and trehalose content declines so that at 7% NaCl (mass/vol.) *Hansenula* contains only 10% of the trehalose present when NaCl is absent from the medium. So, in this yeast at least, trehalose accumulation is inhibited, not enhanced, by decreasing environmental water activities.
TABLE 1.1  The trehalose content in reproductive structures of some fungi.
Trehalose content is expressed as % of dry mass.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>STRUCTURE</th>
<th>TREHALOSE CONTENT</th>
<th>REFERENCE</th>
</tr>
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<td>spore</td>
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<td>Mathre 1969</td>
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<tr>
<td>sativum</td>
<td>spore</td>
<td>2.7</td>
<td>Mathre 1969</td>
</tr>
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1.3 TREHALOSE AS AN OSMOREGULATORY AND CRYPTOPROTECTIVE COMPOUND

1.3.1 Bacteria

Although widespread in fungi, the synthesis of trehalose is considered rare in bacteria (Elbein 1974). However it is in reference to bacteria that most reports claiming trehalose as an osmoregulatory solute are found.

Under certain conditions Escherichia coli responds to an water stress by synthesizing and accumulating trehalose (Roller and Anagnostopoulos 1982; Strøm et al. 1986; Larsen et al. 1987). In addition to trehalose, E.coli may also accumulate K⁺, glutamic acid and other compatible solutes such as glycine betaine when growing in low water activity medium. (Epstein 1986; Le Rudulier et al. 1984; Larsen et al. 1987).

Whether or not trehalose is synthesized when E.coli is adapting or growing in medium of decreased water activity is dependent on the composition of the medium. Le Rudulier and Bouillard (1983) showed that a low level of glycine betaine (1mM) supplied exogenously stimulates the growth of E. coli in low water activity, nutritionally minimal medium. Biochemical studies revealed that glycine betaine transport is osmotically regulated; glycine betaine transport activity is evident only in cells exposed to a water stress (Perroud and Le Rudulier 1985). Other betaines and their precursors also stimulate growth of water stressed E. coli and other members of the Enterobacteriaceae. These include proline, choline and glycine betaine aldehyde. The activation of the betaines and precursors transport systems by a water stress enables the available organic solutes to be accumulated to levels commensurate with the magnitude of the stress. In the presence of these osmoregulatory solutes in the medium, trehalose is not synthesized. In their absence (minimal medium) E. coli responds to an water stress by synthesizing
trehalose (Strøm et al. 1986) with the trehalose content increasing with increasing medium salt concentration. Together with glutamic acid, trehalose is the major organic osmoregulatory solute in *E.coli* under these conditions (Larsen et al. 1987).

Two *E. coli* mutants defective in trehalose synthesis are osmotically sensitive in minimal medium. Strøm et al. (1986) introduced a *galU* mutation into *E. coli*. Because it has defects in the gene coding for glucose-1-phosphate uridylyltransferase, this mutant cannot synthesize UDP glucose (a precursor of trehalose) nor utilize galactose as a substrate (Gal-). In contrast to the parental strain, this mutant is osmotically sensitive, being unable to grow in minimal medium with 0.45M NaCl. A second group of osmotically sensitive, Gal+ mutants (Tn 10 mutants) has also been isolated, none of which are able to accumulate trehalose under water stress. However if the minimal medium is supplemented with glycine betaine the mutants are no longer osmotically sensitive. It is possible that the Tn 10 mutants may be defective in the gene coding for one of the enzymes essential for trehalose biosynthesis, trehalose-6-phosphate synthase (H Gioever and AR Strøm unpublished data, cited in Strøm et al. 1986.)

Although trehalose has been reported in other bacteria besides *E. coli* its function in many of these is less clear. In *Micrococcus* sp. trehalose is found in cells grown in the presence of glucose (Ahmad et al. 1980), there being only minor variations in trehalose content throughout the growth cycle. Unlike yeast, the function of trehalose in micrococci is probably not that of a storage material; Burleigh and Dawes (1967) established that the survival of *Sarcina lutea* (i.e. *Micrococcus lutea*) is not enhanced by the presence of a compound which Ahmad et al. (1980) tentatively identified as trehalose. Evidence that trehalose is an osmoregulatory solute in micrococci is limited to the observation that *M. lutea* accumulated more trehalose
when the water activity of the medium is lowered by increasing the concentration of glucose (Ahmad et al. 1980).

Trehalose is a major carbohydrate in many root nodule bacteria including the slow-growing *Rhizobium* (Streeter 1985) and *Frankia* (Lopez et al. 1984). Its concentration is influenced by medium composition, growth stage of the bacteria and the bacterial strain. Although trehalose has been reported to accumulate under two types of conditions in *Rhizobium*, namely in senescing legume nodules where it is synthesized by bacteroids (Streeter 1981) and in glutamate-containing medium with accumulation ceasing after glutamate depletion, no direct evidence supports the suggestion of Streeter (1985) that trehalose is an osmoregulatory compound in *Rhizobium*.

1.3.2 Cyanobacteria

An osmoregulatory role for trehalose in cyanobacteria was first reported by Reed and Stewart (1983) for *Rivularia atra*. Although earlier studies with the halophilic *Aphanotoche halophytica* (*Synechococcus* sp.) by Miller et al. (1976) had suggested that cyanobacteria respond to changes in external water status by adjusting their intracellular K⁺ concentration, only minor changes in K⁺ concentration in response to salinity were measured in *Synechococcus* and other strains of cyanobacteria by Borowitzka et al. (1980), Mohammad et al. (1983) and Reed et al. (1984). It is the intracellular content of organic compounds that show major variations in response to a salt stress, demonstrating that these compounds, rather than inorganic ions, play a major role in maintenance of positive turgor under conditions of salt stress. Surveys of a large number of cyanobacteria from aquatic habitats of varied ionic composition by Erdmann (1983), Mackay et al. (1983, 1984), Reed et al. (1984) and Reed and Stewart (1985) illustrated that a single low molecular weight organic compound is accumulated as the principal solute in
osmotically-stressed cells. This osmoregulatory solute may be sucrose, trehalose, glucosylglycerol, glycine betaine or glutamate betaine.

In addition to their major solute, one of the other osmoregulatory compounds found in cyanobacteria may also accumulate in some isolates when exposed to a water stress (Mackay et al. 1983, 1984; Warr et al. 1985). This second organic solute normally plays a minor role in maintenance of positive turgor pressure but under certain environmental conditions production of a minor osmoregulatory solute may be increased. For instance, *Spirulina platensis*, initially regarded as an glucosylglycerol accumulator, accumulates trehalose in addition to glucosylglycerol when grown at elevated temperatures and high salinities. The trehalose to glucosylglycerol ratio increased from 1:10 at 20°C to 1:2 at 37°C when grown in 100% saltwater medium (Warr et al. 1985a). The presence of two osmotically important carbohydrates at high temperatures is not confined to cyanobacteria. Muller and Wegmann (1978) reported that *Dunaliella tertiolecta* synthesized sucrose in addition to glycerol when cells are exposed to a water stress at high temperatures.

On the basis of their major osmoregulatory solute and their degree of salt tolerance, Reed et al. (1986) have divided cyanobacteria into three groups. In general trehalose or sucrose is accumulated by the salt-sensitive freshwater and brackish isolates. Cyanobacteria of intermediate halotolerance (marine isolates) accumulate glucosylglycerol while the most halotolerant strains from hypersaline environments accumulate betaines. These three groups of cyanobacteria are not mutually exclusive, however. For example some marine isolates accumulate trehalose (Reed and Stewart 1983), trehalose normally being an osmoregulatory solute in fresh water or brackish isolates. Thus, as suggested by Reed et al. (1986), other factors in addition to the type of osmoregulatory solute govern the degree of halotolerance of cyanobacteria. This is reminiscent of yeast where in both xerotolerant and
nonxerotolerant *Saccharomyces* species glycerol is the major osmoregulatory solute (see section 1.2.2.2).

### 1.3.3 Anhydrobiotic organisms

In 1702 Antonie van Leeuwenhoek made the first observations on the activity of 'animalcules' (rotifers) in gutter-sand and dirt which had been kept in a dried state before being rehydrated many months later. While in a dry state the 'animalcules' seemed to be inert and lifeless but they were observed to be alive and active after being suspended in boiled water. Now it is well established that organisms such as rotifers that do not show visible signs of life under certain conditions are not dead but are in a state known as cryptobiosis (Keilin 1959). In this state metabolic activity is suspended (Hinton 1960; Clegg 1973) and any chemical processes that occur are entirely adventitious (Hinton 1968). Cryptobiotes are able to return to normal life and activity when environmental conditions are more favourable. The ability of an organism to revive from the cryptobiotic state when suitable conditions arise is dependent on the environmental conditions and often viability decreases with the time spent in the cryptobiotic state (see for example Sneath 1962).

The term anhydrobiosis was introduced by Giard (1894) to describe a common kind of cryptobiosis which is induced by the removal of water from the organism. Anhydrobiotic organisms can persist in a dry state for many years but once they are rehydrated normal metabolic activities resume. Two distinct groups of organisms exhibit anhydrobiosis (Crowe 1971). One group consists of those capable of anhydrobiosis only in their early ontogenetic stages or as propagules. A wide range of taxa, including angiosperms (seeds), bacteria (spores) and certain fish (eggs), have an anhydrobiotic stage during their life cycle. The second group of organisms, typified by Leeuwenhoek's 'animalcules', is capable of anhydrobiosis during any stage of their life cycles.
Trehalose is found at particularly high concentrations (as much as 20 percent of dry mass) in a variety of anhydrobiotic organisms capable of surviving dehydration. Typical among these are the macrocyst of the slime mold *Dicotyostelium* (Clegg and Filosa 1961), brine shrimp cysts (Clegg 1965), spores of certain fungi (Sussman and Lingappa 1959) and *Streptomyces antibioticus* (Martin et al. 1986), dry larvae and adults of several species of nematodes (Madin and Crowe 1975) and dry active baker's yeast (Payen 1949).

It has been proposed that anhydrobiotic organisms survive dehydration because trehalose alters the physical properties of membrane phospholipids in the dry state in ways that maintain membrane structure and function (Crowe et al. 1984b). The polar head groups of membrane phospholipids are normally hydrated and the head groups are separated from each other by water molecules (Hauser et al. 1981). When progressively dehydrated, the packing density of the phospholipid head groups rises thereby increasing opportunities for van der Waals interactions among the hydrocarbon chains. By dehydrating a model membrane (calcium-transporting microsomes from muscle) Crowe and Crowe (1982) illustrated the existence of these interactions, which are manifested as lipid phase separations and phase transitions. When rehydrated the membranes are unable to transport calcium and morphological damage is evident (Crowe et al. 1983).

Crowe and Clegg (1973) hypothesized that trehalose preserves membrane structure by hydrogen bonding to the phospholipid headgroups of the dry membrane. These hydrogen bonds may replace the same or similar hydrogen bonds between the lipid and water. Effectively, trehalose is thought to replace bound water thereby stabilizing dry membranes by preventing lipid phase separations and phase transitions. Direct evidence of the "water replacement" hypothesis is still lacking.
Crowe and Clegg's "water replacement" hypothesis predicts that other carbohydrates besides trehalose should be able to stabilize dry membranes. Crowe et al. (1984a) tested the effectiveness of a number of commonly occurring carbohydrates in preserving the structural and functional integrity of Ca-transporting microsomes when exposed to low water activities. Trehalose was shown to be far superior in this regard than other sugars and sugar alcohols (including glycerol). If dehydrated in the presence of trehalose at concentrations of at least 20% of the dry weight of the membranes (similar to physiological levels in anhydrobiotic organisms), phase separations and transitions are not observed and rehydrated membranes are morphologically and functionally similar to freshly prepared membranes. Using a different membrane system, Madden et al. (1985) also concluded trehalose is more effective than sucrose, maltose, glucose and lactose in maintaining the integrity of large unilamellar vesicles subjected to dehydration and rehydration. The ability of the various carbohydrates to preserve membrane structure in the dry state is positively correlated with their relative efficiencies in decreasing the transition temperature, (temperature at which the hydrocarbon chains undergo a change from gel to liquid crystalline state), in interacting with polar phosphate head groups of phospholipids and in expanding monolayer films (Crowe et al.1984a, 1984c).
CHAPTER TWO: MATERIALS AND METHODS
2.1 REAGENTS

The reagents used in this study are listed below.

2.1.1 Media

**Amino acids**: L-histidine, DL-methionine DL-tryptophan (Sigma).

**Carbon sources** (analytical reagent (AR) grade): ethanol, glucose, maltose (Univar); glycerol (BDH).

**Growth factors**: p-aminobenzoic acid, biotin, folic acid, calcium pantothenate, myo-inositol, nicotinic acid, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride (Sigma).

**Salts** (AR grade): calcium chloride dihydrate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride (Univar); calcium chloride hexahydrate (BDH); magnesium sulphate heptahydrate (BDH).

**Trace elements** (AR grade): boric acid, cupric sulphate pentahydrate, citric acid, ferric chloride hexahydrate, ammonium sulphate, manganese sulfate tetrahydrate, potassium iodide (BDH); sodium molybdate dihydrate (Mallinckrodt); zinc sulfate heptahydrate (Univar).

**Fermentor additives**: safflower oil (cold pressed, Healthries of New Zealand Ltd, Auckland, New Zealand), potassium hydroxide (AR, May and Baker); sulphuric acid (AR, Univar).

**Others**: bactopeptone, malt-extract agar, quarter strength Ringers tablets, yeast extract (Oxoid); agar (grade J2, Davis); glycine betaine (free base, Sigma); 2,4-dinitrophenol (AR, BDH).

2.1.2 Chromatography

**Solvents** (AR grade): acetic acid, butan-1-ol, concentrated ammonia, propan-1-ol, pyridine (Univar).

**Detection**: acetone (AR, Univar); anthrone (Sigma); pararosaniline hydrochloride
(Sigma); silver nitrate (AR, May and Baker); sodium hydroxide (AR, Univar); sodium periodate (BDH).

Standards (AR grade): D-arabitol, glycerol, mannitol (BDH); erythritol (Merck); fructose, glucose, sucrose (Univar); galactose, maltose, trehalose (Sigma).

2.1.3 Analyses

Enzymes: aldehyde dehydrogenase, alcohol dehydrogenase (300U/mg), glycerokinase (Candida mycoderma), lactate dehydrogenase (crystalline, from rabbit muscle), pyruvate kinase (Boehringer).

Biochemicals: adenosine triphosphate, ATP (Boehringer); β-nicotinamide adenine dinucleotide, NAD (grade III, Sigma); β-nicotinamide adenine dinucleotide (reduced form) NADH (grade III, Sigma); phosphoenolpyruvate (tricyclohexylammonium salt, Boehringer);

Others (AR grade): acetic acid, ethanol, sulphuric acid, trichloroacetic acid, tetrapotassium pyrophosphate (Univar); anthrone, triethanolamine buffer (Sigma); magnesium sulphate heptahydrate (BDH).

2.2 YEAST STRAINS

The principal yeast strain used in this study was Saccharomyces cerevisiae, strain Y41, American Type culture Collection (ATCC) number 38531. It has been described previously by Anand and Brown (1968) as a nonxerotolerant yeast.

A number of other strains were also used. Saccharomyces rouxii, strain YA, ATCC 38528, is an xerotolerant yeast (Anand and Brown 1968) as is Pichia miso which was obtained from H. Onishi, Department of Agricultural Chemistry, Kagoshima University, Japan. Four species of Candida, C. albicans, C. krusei, C. parapsilosis and C. glabrata, were supplied by W. Crozier, Wollongong Hospital, Australia. S.
baillii and *P. membranaefaciens* were obtained from J. Pitt, CSIRO Food Research Laboratories North Ryde, Australia. A glycogen accumulation mutant of *S. cerevisiae*, strain 212-244-1A, defective at the *glc1* locus (Pringle 1972) was purchased from the Yeast Genetic Stock Center at Berkeley, California. *S. carlsbergensis* and *S. cerevisiae* var. *ellipsoideus* (SO2 resistant) were from our departmental culture collection. Three species were isolated during the course of this study. They were tentatively identified by W. Crozier at Wollongong Hospital, using Lodder's classification (1970), as a species of *Kloeckera*, a morphological variant of *S. cerevisiae*, and a species of *Torulopsis*.

Stock cultures were maintained on slopes of agar medium and stored in screw capped bottles at 4°C. *S. rouxi* and *P. miso* were maintained on synthetic honey agar slopes while the other strains were maintained on malt extract agar. Stocks were sub-cultured every three months.

### 2.3 MEDIA

Three liquid growth media were used. The composition of basal yeast medium (BYM) has been described by Anand and Brown (1968). The water activity of this medium is 0.997 and the pH was adjusted to 5.9.

Salt broth was prepared by adding 10% NaCl (mass/vol.) to BYM. The water activity of salt broth is 0.936 (Anand and Brown 1968).

Wickerham's (1951) chemically defined medium, later described by Barnett et al. (1983) was prepared as a 10X concentration solution and, after being filter-sterilized, it was kept in the refrigerator and used as needed. Unless otherwise stated the carbon source present in Wickerham's medium was glucose. Glucose was
omitted from the 10X concentration medium and the carbon source filter-sterilized separately for those experiments where a carbon source other than glucose was being used. The concentration of carbon source in Wickerham's medium was always 0.5%, mass/vol.

The agar media used were: malt extract agar (MA); synthetic honey agar (SHA) containing 1.0% yeast extract (mass/vol.), 2.0% agar (mass/vol.) and 48.0% glucose (mass/vol.) unless otherwise stated; salt agar containing BYM, 1.5% agar (mass/vol.) and 10% NaCl (mass/vol.) or as specified. The water activity of SHA containing 48% glucose is 0.924 while that of salt agar containing 10% NaCl is 0.936 (Robinson and Stokes 1965). When required glycerol and glycine betaine were added to salt agar before sterilization.

Except for Wickerham's medium, media were sterilized by autoclaving at a pressure of 83 kPa for 12 minutes or, in the case of SHA, at 69 kPa for 10 minutes.

2.4 GROWTH CONDITIONS

2.4.1 Inoculation procedure

Inocula were grown in an orbital shaker (Gallenkamp) at 30°C and 200 rpm. The initial or preinoculum culture was prepared by transferring a loop of a slope culture to a 100ml Erlenmeyer flask containing 50 ml of the specified growth medium. It was incubated until the cells were in the stationary phase of growth. The second or inoculum culture contained a 5% inoculum, by volume, from the initial culture and the same medium composition as the initial culture. The ratio of medium volume to Erlenmeyer flask volume in the inoculum culture was normally 1:2. Once in stationary growth phase, cells from the inoculum culture were used to inoculate experimental cultures.
The incubation times that were normally used for preinoculum and inoculum cultures of *S. cerevisiae* when growing in BYM and Wickerham's medium are listed in Table 2.1.

**TABLE 2.1 Incubation times for inoculum cultures of *Saccharomyces cerevisiae***

<table>
<thead>
<tr>
<th>CULTURE</th>
<th>MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BYM</td>
</tr>
<tr>
<td></td>
<td>Wickerham's</td>
</tr>
<tr>
<td>preinoculum</td>
<td>24h</td>
</tr>
<tr>
<td>inoculum</td>
<td>16-18h</td>
</tr>
<tr>
<td></td>
<td>48h</td>
</tr>
</tbody>
</table>

The incubation times for the other yeasts are specified with the relevant figures in the results chapter. Glucose was the only added carbon source present in preinoculum and inoculum cultures.

### 2.4.2 Batch culture experiments

Generally the inoculum cultures used to inoculate experimental cultures were of the same medium composition as the experimental cultures, the exceptions being non-proliferating, salt adaptation and carbon source experiments. The volume of the inoculum was 5% of the final experimental culture volume (v/v) unless otherwise specified.

For non-proliferating experiments all the inoculum culture was used to initiate an experimental culture, with the volume of the inoculum and experimental culture being the same. The 72 h inoculum culture was harvested by aseptic filtration through a 0.8 μm membrane filter. The filter and cells were placed in fresh
Wickerham's medium devoid of glucose and, in some experiments, containing 2,4-dinitrophenol. Dinitrophenol was added as an ethanolic solution to reach a final concentration of 2mM; it was added to the medium just before use.

In salt transfer experiments a 0.5% inoculum (v/v) was used to inoculate a fresh flask of BYM. At the specified time after incubation the culture was harvested aseptically (centrifugation at 0°C for 3 minutes at 8000g) and the cells resuspended in salt broth so that the final cell concentration was between $2 \times 10^6$ and $4 \times 10^6$ c.f.u./ml.

Experimental cultures, like the preinoculum and inoculum, were grown in an orbital incubator in Erlenmeyer flasks, the flask volume being twice the volume of the medium. The incubation temperature was normally 30°C.

2.4.3 Fermentor experiments
A one litre (final volume) fermentor was used (LH Series III, LH Fermentation, Stoke Poges, Bucks, England) for continuous culture and anaerobic batch experiments. The pH was maintained at 5.9 ±0.1 by the automatic addition of 2M KOH and 2M H$_2$SO$_4$ and temperature was controlled at 30°C. The antifoam used was an unrefined, sterilized, vegetable oil. Incoming air was passed through two membrane filters of 0.2 μm pore size connected in series. Dissolved oxygen content of the medium was measured by an oxygen electrode. An aeration rate of 3 fermentor volumes of air/min ensured that the oxygen content of the medium was 65% saturated. The volume within the fermentor was kept constant by using an overflow weir (5 mm diameter). Agitation was achieved by means of flat bladed impellers operating at 700rpm. Samples of the the culture were removed from the vessel via an air-lock device into a sterile screw-top bottle. The culture vessel was shielded from the light.
The assembled vessel was sterilized by autoclaving at 83 kPa for 45 minutes. The medium reservoir, containing 20l of BYM, was autoclaved separately for 80 minutes. The chemostat was erected and filled with sterile medium to which was added a 1% inoculum (v/v) of *S. cerevisiae*. It was operated batch-wise until the cells reached the stationary phase of growth after which medium was pumped in by a variable speed peristaltic pump.

For some experiments the fermentor was operated anaerobically during the batch culture phase. Anaerobic conditions were achieved (as determined by oxygen electrode readings) by purging the culture for two hours before inoculation and throughout the fermentation with oxygen-free grade nitrogen (Commonwealth Industrial Gases) at the rate of 3 fermentor volumes/min. The pH was not controlled during anaerobic batch culture experiments.

Continuous culture using BYM was carried out according to the chemostatic principle (Monod 1950; Novick and Szilard 1950) with glucose as limiting substrate. Steady states were tested for glucose limitation by monitoring cell density x (measured as dry weight and cell numbers) after the addition of 5% glucose (mass/vol.). The working volume V was kept constant at 488 ml. The dilution rate D was varied by changing the medium flow rate F (D = F/V). The course of the cultivation was followed by measuring dry weight and taking total cell counts. Steady states were generally reached within ten mean generation times (g = 0.693/D) after a change in flow rate F (without intervening disturbances of the cultivation). At steady states the value for specific growth rate μ (μ = 1/x.dx/dt) of the population is identical with the adjusted dilution rate (D).

In order to avoid disturbing the steady states, samples were removed from continuous cultures after 3 times the volume of the previous sample had been replaced.
2.5 MEASUREMENT OF GROWTH

2.5.1 Growth rate

Yeast growth in liquid culture was measured turbidimetrically at 700nm using a Bausch and Lomb SP20 spectrophotometer (1 cm light path). Exponential growth rates were determined from the changes in optical density, total count (see section 2.5.3.2) and dry mass (section 2.6.2) during exponential growth.

2.5.2 Xerotolerance

The various yeast strains were assessed for xerotolerance by their ability to grow on low water activity agars. Cells from 7 day slope cultures were streaked onto duplicate MA, SHA (containing 30, 42, 44, and 48% glucose,mass/vol.) and salt agar (8% NaCl,mass/vol.) in the manner illustrated in Figure 2.1. After 28 days incubation at 30°C the growth on the plates was recorded using the following criteria: + -for growth only in the first streak; ++ -for growth in first and second streaks; +++ -for growth in first, second and third streaks; ++++ -for growth in all four streaks.

Figure 2.1 Streak method used for the qualitative determination of the degree of xerotolerance.
2.5.3 Counting

2.5.3.1 Sample preparation
Samples were aseptically withdrawn from cultures and routinely cell clumps were disaggregated either by ultrasonic oscillation or homogenization. Several other techniques, including vigorous agitation by a vortex mixer and polytron blender, were ineffective in dispersing clumps. Exposure of a suspension of *S. rouxii* to 2 minutes of ultrasonic oscillation (80W at 50 kHz) was sufficient to dissociate clumps containing 30 or more cells. Subjecting suspensions to 75 double strokes in a glass teflon homogenizer (diameter of 16 mm) was equally effective and, as viability studies showed that *S. cerevisiae* did not survive ultrasonic oscillation, clumps were routinely dispersed by homogenization.

2.5.3.2 Total counts
The cells were counted microscopically (positive phase contrast optics) using a haemocytometer (Weber, 0.1 mm deep). Normally the number of colony-forming units (c.f.u.) was counted, a c.f.u. being defined as any cell or aggregate of cells which, if viable, would form a single colony on agar media. Any c.f.u. that was not a single cell was scored as a budded unit irrespective of the size of the bud or buds. Budded c.f.u. and phase-dark c.f.u. (those c.f.u. that are phase-dark or contain one or more phase-dark cells) were expressed as a percentage of the total number of c.f.u. To ensure that the count determined was within the 95% level of confidence, a minimum of 300 c.f.u. were scored for each sample.

2.5.3.3 Viable counts
In any one experiment only one plating method, the pour plate or drop plate technique, was used to determine viability. This was necessary because, at least for MA, viability values determined with the drop plate method were normally about 13% lower than the values determined with the pour plate method.
Yeast suspensions were serially diluted in sterile quarter-strength Ringer's solution or, in the case of the salt transfer experiments, 10% NaCl (mass/vol.) in preparation for plating either by the pour plate or drop plate method. For adaptation experiments the suspensions were diluted 100-fold in 24% glucose (mass/vol.). After the specified period of adaptation the suspensions were further diluted in the same dilutent and plated.

Agar plates were prepared 24 hours before use. Four drops of the appropriate dilutions, each drop having a volume of about 0.03 ml, were placed onto the agar media. Pour plates were prepared by pipetting 0.1 ml of a cell suspension into a petri dish and mixing with it 10 ml of molten agar (43°C). All viable counts were done in duplicate.

Plates were incubated at 30°C; the low water activity agar plates were incubated in plastic bags to prevent evaporation. Colonies were visible and able to be counted after three days on the high water activity agars (MA and BYM agar); longer incubation periods were required for the low water activity agar plates. Colonies on SHA were counted after 7 days and those on salt agar after 21 days.

MA was routinely used to determine viability at high water activity because viable counts on BYM agar (BYM medium plus 1.5% agar, mass/vol.) gave a value, on average, 10% lower than the MA count.
2.6 ANALYTICAL METHODS

2.6.1 Cell sampling
Samples were withdrawn from cultures and rapidly vacuum-filtered using a cellulose acetate filter (pore size 0.8μm). The cells and filter were frozen in liquid nitrogen, freeze-dried and stored at -80°C until extracted. If the medium contained glucose or maltose and trehalose content was to be estimated, the cells were washed twice on the filter with 2 ml of isotonic buffer to remove residual extracellular hexose. This buffer contained the major salts present in Wickerham's medium: KH₂PO₄ (850 mg), K₂HPO₄ (150 mg), MgSO₄.7H₂O (500 mg), NaCl (100 mg) and CaCl₂.6H₂O (100 mg) made up to 1l with distilled water. The filtrate (cell-free medium) was also frozen in liquid nitrogen after being placed in an airtight plastic vial and was stored at -80°C before extracellular ethanol, glucose and glycerol determinations were made.

2.6.2 Dry mass
Dry mass was determined by trapping cells on tared 0.8μm membrane filters. The cells were washed on the filters with 10 ml of distilled water. After drying at 105°C for 24 hours, the filters were cooled in a dessicator and weighed.

2.6.3 Cell extraction
Freeze-dried yeast was extracted with ethanol after the method of Edgley and Brown (1983). For comparative purposes trehalose was also extracted with trichloroacetic acid (0.5 M; Trevelyan and Harrison 1952). For both ethanol and trichloroacetic acid extractions approximately 1 ml of ethanol or trichloroacetic acid was added per 4 mg of dry mass of yeast. After extracts were dried by rotary evaporation under vacuum at 40°C, they were resuspended in 1ml of H₂O and stored at -80°C.
2.6.4 Thin-layer chromatography

Silica gel G thin-layer chromatography (TLC) plates from Analtech Inc. were activated at 110°C for 12 hours before use. Separation of polyols was achieved by chromatography in butan-1-ol + acetic acid + water (6:1:2 by volume) or propan-1-ol + concentrated NH₄OH + water (6:2:1 by volume). A third solvent system, butan-1-ol + pyridine + water (15:3:2 by volume) was used for separating trehalose from other carbohydrates. Ten µl of 1% standard polyol and sugar solutions (mass/vol.) were co-chromatographed with the extracts. After being developed for about three hours the plates were dried overnight before compounds were visualized with alkaline silver nitrate reagent (Trevelyan et al. 1950), periodate-Schiff's base reagent (for polyols) (Baddiley et al. 1956) and anthrone reagent (Block et al. 1958). Identification of compounds was based on their reactions with these reagents and their chromatographic mobilities in relation to known compounds in the three solvent systems.

When the polyol contents of the various yeasts were to be compared, the volume of extract spotted on the TLC plates was measured an extract volume equivalent to approximately 5 mg of dry yeast was used for each strain.

2.6.5 Ethanol estimations

The enzymic method described by Beutler (1984) was used to estimate ethanol.

2.6.6 Glucose estimations

Glucose was estimated enzymically using a commercial glucose oxidase kit (GOD-Perid, Boehringer) and the method described by Edgley and Brown (1983).

2.6.7 Glycerol estimations

Eggstein's and Kuhlmann's method (1974) for the enzymic estimation of glycerol was modified in two ways. The final concentration of NADH was reduced from 0.4
mM to 0.1 mM (Edgley and Brown 1983) and MgCl$_2$.6H$_2$O was replaced by MgSO$_4$.7H$_2$O.

2.6.8 Trehalose estimations

Trehalose was estimated using anthrone (Stewart 1975). The overall volume of the reaction mixture was reduced by a factor of five: 0.2 ml of cell extract and 1.0 ml of anthrone reagent was used in each determination.

2.6.9 Spectrophotometers

Absorbances for trehalose and glucose estimations were measured using a Gilford Stasar III spectrophotometer (1 cm light path). The Zeiss PMQ II spectrophotometer (1 cm light path), fitted with a recorder, was used for glycerol and ethanol analyses. All analyses were done in duplicate.
CHAPTER THREE: RESULTS
3.1 WATER ACTIVITY AND VIABILITY

3.1.1 Transfer of *Saccharomyces cerevisiae* to salt broth

There was a marked decrease in viability on MA when exponentially growing *Saccharomyces cerevisiae* was transferred from BYM to salt broth (figure 3.1). In a similar experiment Edgley and Brown (1983) also found a decrease in MA viability of similar magnitude. They showed that after about 50 h in salt broth with the MA and SHA count gradually recovered, the MA count achieving pre-transfer levels at about 140 h. In the present experiment the high percentage of phase-dark c.f.u. 50 h after transfer (figure 3.1) was similar to the pre-transfer level and this, together with the rising number of budded c.f.u., indicated that the yeast population was entering that stage of the adaptation process characterized by rising viable counts on both types of plating medium. Up until this stage of the adaptation no culture growth had occurred as seen by the invariant total count. Also the SHA count was constant after stress exposure and was consistently about 3 log units lower than the total count.

The response of the population of *S. cerevisiae* was totally different if stationary phase rather than exponential phase yeast were transferred to salt broth (figure 3.2). The adaptation of stationary phase yeast was characterized by a lag phase lasting about 50 h followed by an exponential growth period in which there was fairly good agreement between viable and total counts. The exponential growth rate was approximately 0.08 generations/h which was about one sixth of that in BYM (0.46 generations/h, calculated from figure 3.3).

As described in section 2.4.2 the quantity of yeast transferred to salt broth was approximately $3 \times 10^6$ c.f.u./ml (a log value of 6.5) in both exponential and stationary phase transfer experiments. Thus, as expected, the zero time total and MA counts were similar in the two experiments but the counts on SHA were
FIGURE 3.1 Adaptation of *Saccharomyces cerevisiae* to salt broth after the transfer of exponential phase cells from BYM.

Top panel: proportion of the total c.f.u. that are

- phase-dark
- budded

Bottom panel:

- total count
- MA count
- SHA count

The points represent the mean of two experiments (mean of the log values for total and viable counts).

The cells were transferred to the salt broth (containing 10% NaCl) after growing for 5 hours in BYM.
FIGURE 3.2 Adaptation of *Saccharomyces cerevisiae* to salt broth after the transfer of stationary phase cells from BYM.

Top panel: proportion of the total c.f.u. that are
- □ phase-dark
- ○ budded

Bottom panel:
- ○ total count
- ■ MA count
- □ SHA count

The points represent the mean of two experiments (mean of the log values for total and viable counts).

The cells were transferred to the salt broth (containing 10% NaCl) after growing for 15 hours in BYM.
profoundly different, the exponential phase zero time SHA count being only 2% of the corresponding stationary phase count. Note also the SHA count recovery to the level of the MA count in the stationary phase experiment and the absence of any such recovery in the exponential phase adaptation.

3.1.2 Growth of *Saccharomyces cerevisiae* in BYM

3.1.2.1 Water stress plating hypersensitivity

The vastly different zero time SHA viability values established in the salt transfer experiments were investigated further by inoculating *Saccharomyces cerevisiae* into a high water activity medium, BYM and measuring SHA viability during a growth cycle (figure 3.3). During the exponential growth phase the ability of the yeast to form colonies on either SHA or on another low water activity agar, 10% salt agar, was dramatically reduced. The viable count on 10% salt agar was less than the SHA count. On the non-stressing agar, MA, no such reduction in viability was observed. The plating discrepancy \((\log_{10} \text{MA count} - \log_{10} \text{SHA count})\) was at a maximum 5 h after culture inoculation, having a value of 4.52, meaning that 1 c.f.u. in \(3 \times 10^4\) was viable on SHA. This phenomenon is called "water stress plating hypersensitivity" throughout the rest of this thesis.

Water stress plating hypersensitivity correlated not only with the exponential growth phase but also with the period when the percentage of budded c.f.u. was highest (figure 3.3). Because of the inoculation procedure used, in particular the inoculation of the experimental culture with stationary phase yeast that presumably had come to rest in the G1 stage of the cell division cycle, cell division in the experimental culture were likely to synchronized, at least for the first few division cycles. The possibility was entertained that a specific event in the cell division cycle was critically sensitive to a sudden water stress imposed by plating onto low water activity agar, the sensitivity of this event having prevented the completion of the
FIGURE 3.3 Partial growth cycle of *Saccharomyces cerevisiae* in BYM.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:

- □ MA count
- ■ SHA count
- ● 10% salt agar count

The points represent the mean of eight experiments (mean of the log values for total and viable counts), with the vertical bars denoting the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.

Samples were not treated for cell clumping.
cell division cycle on SHA or salt agar. At the time of greatest plating
hypersensitivity (5 h) the culture would have had to be synchronized to such a
degree that all but 1 c.f.u. in $1 \times 10^4$ (the size of the plating discrepancy) were at
the same point in the cell division cycle. Judging from the proportion of unbudded
c.f.u. (see figure 3.3), about two-thirds of the yeast population was in G1 at the
time of inoculation and so, at the most, two-thirds of the population were dividing
synchronously. Thus at the time of greatest plating hypersensitivity only two thirds
of the yeast population were at the same point in the cell division cycle. As a much
greater proportion of the population than two-thirds was hypersensitive at 5 h,
water stress plating hypersensitivity was evidently not caused by the sensitivity of a
particular event in the cell division cycle.

3.1.2.2 Prolongation of the exponential growth phase

Water stress plating hypersensitivity is a phenomenon peculiar to exponentially
growing yeast. *Saccharomyces cerevisiae* was resistant to plating on a low water
activity agar during the periods of non-proliferation in the growth cycle, that is
during the lag and stationary phases. The addition of glucose to a culture in the lag
phase extended the duration of the exponential phase and with it lengthened the period
of plating hypersensitivity in *S. cerevisiae* (figure 3.4).

Normally cultures were inoculated with a 5% inoculum. Reduction in the size of the
inoculum lengthened both the exponential growth phase and the period of water
stress plating hypersensitivity (figure 3.5, compared to figure 3.3).

Extension of the exponential growth phase was also achieved by incubating an
experimental culture of *S. cerevisiae* at the suboptimal temperature of 15°C (figure
3.6). The biphasic nature of the growth cycle (diauxie) was evident in this
experiment. Corresponding with the two periods of exponential growth were two
periods of water stress plating hypersensitivity. With a growth rate of 0.17
FIGURE 3.4 Effect of lag phase addition of glucose on water stress plating hypersensitivity in *Saccharomyces cerevisiae*.

The experiment was conducted in BYM.

Top panel: viability without the addition of supplementary glucose.

Bottom panel: viability when 5g of glucose per litre of medium was added at the time indicated by the arrow.

For both panels:

- MA count
- SHA count

Samples were not treated for cell clumping.
FIGURE 3.5 Effect of a 0.5% inoculum on water stress plating hypersensitivity in *Saccharomyces cerevisiae*.

The experiment was conducted in BYM.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:

- □ total count
- ■ MA count
- • SHA count

The points represent the mean of two experiments (mean of the log values for total and viable counts).

Samples were not treated for cell clumping.
FIGURE 3.6 A growth cycle of *Saccharomyces cerevisiae* in BYM at 15°C.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- □ total count
- □ MA count
- • SHA count

Note that the time scale is different from that in the previous figures.
generations/h the first exponential phase lasted about 24 h followed by a transient period resembling stationary phase (depicted by a higher SHA count at 24 h). The onset of the second exponential growth phase was marked by an increase in the proportion of budded c.f.u. at 30 h and a decrease in the SHA count. The difference in the size of the plating discrepancies between the first and second exponential phases may reflect the different growth rates of the two phases; in this experiment at least there was a positive correlation between growth rate and maximum plating discrepancy. Comparison of these results with the values from the exponential growth phase at 30°C (table 3.1) revealed that the plating discrepancy in the first exponential phase at 15°C was much higher than expected if growth rate alone was responsible for the size of the plating discrepancy.

The two different exponential growth rates in the diauxic growth cycle at 15°C presumably reflect the primary carbon sources being consumed in these growth

**TABLE 3.1** A comparison between exponential growth rate and plating discrepancy in *Saccharomyces cerevisiae* growing in BYM.

These values were calculated from figures 3.3 and 3.6.

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>EXPONENTIAL GROWTH RATE</th>
<th>MAXIMUM PLATING DISCREPANCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp (°C)</td>
<td>Exp. growth phase</td>
<td>(generations/h)</td>
</tr>
<tr>
<td>30</td>
<td>1st</td>
<td>0.56</td>
</tr>
<tr>
<td>15</td>
<td>1st</td>
<td>0.17</td>
</tr>
<tr>
<td>15</td>
<td>2nd</td>
<td>0.06</td>
</tr>
</tbody>
</table>

phases, namely glucose in the first exponential growth phase and ethanol in the second exponential growth phase. The influence of the type of carbon metabolism on
water stress plating hypersensitivity was examined in a series of experiments described in section 3.2.

3.1.2.3 Agar supplementation and water stress plating hypersensitivity

The addition of millimolar quantities of glycine betaine to low water activity agar permits *Escherichia coli* to grow at otherwise inhibitory levels of water activity (Le Rudulier et al. 1984). It also enhances growth in several strains of *Rhizobium* when grown under salt stress (Le Rudulier and Bernard 1986). Glycine betaine is transported into these bacteria where it functions as an osmoregulatory solute. The presence of glycine betaine in 8% salt agar did not, however, improve the viability of *Saccharomyces cerevisiae* on this agar (figure 3.7).

As glycerol (rather than glycine betaine) functions as an osmoregulatory solute in *S. cerevisiae* and Anand (1969) reported that some xerotolerant yeasts could grow at lower water activities when glycerol was added to the medium, the viability of *S. cerevisiae* during growth in BYM was measured using salt agar containing two concentrations of glycerol (figure 3.7). Glycerol in the agar did not have a protective effect on yeast viability and, because it lowered further the agar water activity, the viable counts were below those obtained just with 8% salt agar. The viability on 8% salt agar containing 1M glycerol was so low during mid-exponential growth that no colonies grew on plates seeded with undiluted yeast suspension. This value is plotted in figure 3.7 as log$_{10}$ 0.0 to indicate this.

The relation between the exponential phase viable count and the water activity of the stressing agar is illustrated in figure 3.8. The difficulty *S. cerevisiae* had in growing on the lower water activity agars was shown by the small colonial size. Those colonies that grew on 8 and 9% NaCl agar were very small (less than 1 mm in diameter) and did not enlarge on prolonged incubation.
FIGURE 3.7 Viable counts of *Saccharomyces cerevisiae* on salt agar containing glycine betaine or glycerol during a partial growth cycle in BYM.

Top panel:
- ■ 8% salt agar + 0.5M glycerol
- ● 8% salt agar + 1.0M glycerol

Bottom panel:
- ▲ 8% salt agar + 0.25 mM glycine betaine
- ■ 8% salt agar + 0.50 mM glycine betaine
- ● 8% salt agar

Samples were not treated for cell clumping.
The results in figure 3.3 indicated that the nature of the solute used to adjust agar influenced the size of the plating discrepancy: agar containing NaCl adjusted to 0.943aw (calculated from Harris 1981) was more inhibitory than agar adjusted to a lower water activity (0.924) with glucose. A quantitative comparison between the count on SHA and salt agar of the same water activity was possible from the experiment illustrated in figure 3.8. When SHA was used the viable count was $1.26 \times 10^4$ colonies/ml. By extrapolating from the linear regression line drawn in figure 3.8 the viable count on salt agar having the same water activity as SHA (0.924) is calculated to be 2 colonies/ml.

3.1.2.4 Acquired resistance to low water activity agar

It has been frequently demonstrated (see section 1.2.1.3) that yeasts can adapt and grow at water activities below those that are normally tolerated if they are exposed to increasingly lower water activities in a stepwise fashion. *Saccharomyces cerevisiae* was grown in continuous culture to investigate the relation between growth rate and plating discrepancy (see section 3.3.2). Yeast taken from steady-state continuous cultures was allowed to adapt to an intermediate water activity for various time intervals prior to plating. The adaptation pattern is illustrated in figure 3.9.

3.1.3 The occurrence of water stress plating hypersensitivity in other yeast

3.1.3.1 *Saccharomyces rouxii*

*Saccharomyces rouxii*, which is xerotolerant, did not display water stress plating hypersensitivity when grown in BYM (figure 3.10). Its exponential growth rate was lower than that of *S. cerevisiae* (0.29 compared to 0.46 generations/h) so the exponential growth period was longer, being sustained for more than 12 h in *S.
FIGURE 3.8 Plate counts of exponential phase *Saccharomyces cerevisiae* as a function of agar NaCl concentration.

The experiment was conducted in Wickerham's medium.

The points represent the mean of the log values from three experiments with the vertical bars denoting the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.

The dashed line represents the calculated linear regression line.

The water activity values were calculated from the data of Robinson and Stokes (1965).
Samples were taken from a steady state continuous culture having a dilution rate of 0.055h⁻¹.

- MA count
- SHA count

The points represent the mean of the log values from two experiments.
rouxii. The initial lag phase was not evident in this yeast as it was in S. cerevisiae (figure 3.30) whereas the apparent increase in growth rate after 8 h growth was unique to the S. rouxii growth cycle.

In S. rouxii the viable count was consistently lower on the salt agar than on the other stressing agar, SHA. Evidently, as was the case with S. cerevisiae, NaCl was more inhibitory to colonial growth than glucose.

3.1.3.2 Other species

Before screening for water stress plating hypersensitivity in yeasts from the Saccharomyces genus and other genera, their degree of xerotolerance was assessed (table 3.2). For comparative purposes S. cerevisiae (classified as a nonxerotolerant species) and S. rouxii (a xerotolerant species) are included in the table. As Pichia miso is known to be a xerotolerant yeast (Onishi 1963) its xerotolerance was not assessed.

Except for Candida albicans all the Candida species were salt rather than sugar tolerant. Their sugar tolerance was similar to S. cerevisiae but S. cerevisiae was much more salt sensitive than the Candida species. The tolerance profiles of S. cerevisiae var. ellipoideus and S. carlbergensis most closely resembled that of the nonxerotolerant S. cerevisiae while, based on the similarity of their tolerance profiles to S. rouxii, S. baillii and C. albicans could be classified as xerotolerant yeasts.

The yeasts were screened for water stress plating hypersensitivity by measuring viability on SHA during exponential growth in BYM. Table 3.3 lists those that displayed plating hypersensitivity and those that did not. In addition, figures 3.11, 3.12, 3.13 and 3.14 illustrate water stress plating hypersensitivity in the four sensitive yeasts, two of which were strains of S. cerevisiae.
FIGURE 3.10 Partial growth cycle of *Saccharomyces rouxii* in BYM.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- ■ total count
- ◆ MA count
- ● SHA count
- ▲ 8% salt agar

The points represent the mean of two experiments (mean of the log values for total and viable counts).

The preinoculum culture was grown for 24 hours, the inoculum culture for 17 hours and a 2% inoculum was used to initiate the experimental culture.
Log 10 (count/ml)

Incubation time (h)

Incubation time (h)

Budded c.f.u. (%)

Incubation time (h)
TABLE 3.2 Growth of selected yeasts on low $a_w$ plating media.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>MA</th>
<th>SHA (% glucose)</th>
<th>8% salt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>42</td>
<td>44</td>
</tr>
<tr>
<td>C. albicans</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. krusei</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kloeckera</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P. membranaefaciens</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. baillii</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. cerevisiae var.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ellipsoideus</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. carlsbergensis</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Torulopsis</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Growth scoring system: see section 2.5.2. In brief: 0 = no growth; + = minimal growth, with increasingly better growth indicated by additional +; - = growth not determined.

The other two sensitive yeasts, a species of Kloeckera and C. krusei were more tolerant of salt agar than SHA (table 3.2). Candida was unable to grow on SHA containing 48% glucose so it was tested for hypersensitivity by plating onto SHA containing 42% glucose. Even on this higher water activity agar the maximum plating discrepancy ($\log_{10}$ MA count - $\log_{10}$ 42% glucose SHA count) was 4.62 (figure 3.13), 0.10 larger than the maximum plating discrepancy ($\log_{10}$ MA count - $\log_{10}$ SHA (48% glucose) count) obtained for S. cerevisiae (see figure 3.3). For Kloeckera the maximum plating discrepancy, lower than either the S. cerevisiae
or *C. krusei* values, was 3.85 (figure 3.14). *Kloeckera* was isolated from a *S. cerevisiae* salt transfer experiment where its faster growth rate distinguished it from *S. cerevisiae*.

**TABLE 3.3** Division of selected yeasts into those that displayed water stress plating hypersensitivity and those that did not.

<table>
<thead>
<tr>
<th>TOLERANT SPECIES</th>
<th>SENSITIVE SPECIES</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em></td>
<td><em>Candida krusei</em></td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td><em>Kloeckera</em></td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td><em>Pichia membranaefaciens</em></td>
<td><em>S. cerevisiae var. ellipsoideus</em></td>
</tr>
<tr>
<td><em>Pichia miso</em></td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces baillii</em></td>
<td></td>
</tr>
<tr>
<td><em>S. carlsbergensis</em></td>
<td></td>
</tr>
<tr>
<td><em>S. rouxii</em></td>
<td></td>
</tr>
<tr>
<td><em>Torulopsis</em></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 3.11 Partial growth cycle of *Saccharomyces cerevisiae* var. *ellipsoideus* in BYM.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:

△ total count  ■ MA count  ● SHA count

The preinoculum and inoculum cultures were grown for 24 hours. The experimental culture was initiated by a 2% inoculum.

FIGURE 3.12 Partial growth cycle of a morphological variant of *Saccharomyces cerevisiae* in BYM.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:

■ total count  □ MA count  ● SHA count

The inoculation schedule in table 2.1 was used. The experimental culture was initiated by a 2% inoculum.
FIGURE 3.13 Partial growth cycle of *Candida krusei* in BYM.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- total count
- MA count
- SHA (42% glucose) count

The points represent the mean of two experiments (mean of the log values for total and viable counts).

The preinoculum and inoculum cultures were grown for 24 hours.
The experimental culture was initiated by a 2% inoculum.

FIGURE 3.14 Partial growth cycle of *Kloeckera* in BYM.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- total count
- MA count
- SHA count

The points represent the mean of two experiments (mean of the log values for total and viable counts).

The preinoculum and inoculum cultures were grown for 24 hours.
The experimental culture was initiated by a 2% inoculum.
3.2 WATER STRESS PLATING HYPERSENSITIVITY AND UTILIZATION OF CARBON

3.2.1 Diauxic growth curve

Exponential growth of *Saccharomyces cerevisiae* in BYM at 15°C was distinguished by two exponential growth phases separated by a lag phase (figure 3.6). A similar type of diauxic growth curve at 30°C is illustrated in figure 3.15a. The exponential growth rate in the second exponential phase (0.074 generations/h) was approximately one sixth of that in the first exponential phase (0.46 generations/h).

Glucose was primarily fermented to ethanol in the first exponential growth phase (figure 3.15b). Very little glucose remained in the medium at 5h, at which point, because the culture was vigorously aerated, utilization of ethanol began. As shown in figure 3.3, viability on low water activity agar was at its lowest after 5h incubation, rising rapidly thereafter.

3.2.2 Alteration of the diauxic growth pattern by aeration

Batch cultivation in the fermentor permitted oxygen content to be monitored and controlled. When the oxygen content of the medium was maintained at 65% saturated, *Saccharomyces cerevisiae* displayed water stress plating hypersensitivity during the first exponential growth phase (figure 3.16). The period of plating hypersensitivity was longer however, than that described for batch experiments in the orbital shaker.

*S. cerevisiae* was prevented from entering the second exponential growth phase, characterized by the aerobic utilization of the fermentation products, by growing the yeast under anaerobic conditions. Glucose was consumed after about 12 h incubation (figure 3.17b) yet the rapid recovery in SHA count that normally occurs upon depletion of glucose in aerobic conditions did not occur under anaerobic conditions.
FIGURE 3.15a Cell yield of *Saccharomyces cerevisiae* during a growth cycle in BYM.

The points represent the means of two experiments.

FIGURE 3.15b Medium glucose concentration and ethanol production and utilization by *Saccharomyces cerevisiae* during a growth cycle in BYM.

△ Glucose
■ Ethanol

The points represent the means of two experiments.

These analyses were performed during the experiment illustrated in figure 3.15a.
Cell yield (mg dry yeast/ml)
FIGURE 3.16 Partial growth cycle of *Saccharomyces cerevisiae* in the fermentor.

The experiment was conducted in BYM.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- ■ total count
- □ MA count
- • SHA count

The points represent the mean of three experiments (mean of the log values for total and viable counts) and the vertical bars denote the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.
FIGURE 3.17a Growth cycle of *Saccharomyces cerevisiae* when grown anaerobically for the first 24 hours of the incubation.

The experiment was conducted in BYM.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- total count
- MA count
- SHA count

The points represent the mean of three experiments (mean of the log values for total and viable counts) and the vertical bars denote the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.

The arrow indicates the time when aerobic conditions began.
The arrow indicates the time when aerobic conditions began.

These analyses were performed during the experiment illustrated in figure 3.17a.

FIGURE 3.17c Extracellular glycerol and ethanol in a *Saccharomyces cerevisiae* culture when grown anaerobically for the first 24 hours of the incubation.

- Glycerol
- Ethanol

The experiment was conducted in BYM.

The arrow indicates the time when aerobic conditions began.
FIGURE 3.18 Partial growth cycle of *Saccharomyces rouxii* in
Wickerham's medium.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- • total count
- △ MA count
- □ SHA count

The preinoculum culture and the inoculum culture were grown for
48 hours and a 2% inoculum was used to initiate the experimental
culture.
The gradual decline in ethanol content during the anaerobic phase of the experiment presumably reflects flushing of ethanol from the fermentor by the gas flow.

As mentioned above, *S. cerevisiae* was more resistant to a water stress in aerobic growth conditions once the medium glucose concentration was below about 2mM (figures 3.3 and 3.15b). Such a correlation between resistance, low glucose concentration and aerobic conditions was not observed when the 24 h anaerobic culture was supplied with air. The onset of aeration coincided with an increase in plating discrepancy (figure 3.17a) despite the absence of glucose in the medium (figure 3.17b). Coincident with this increase were an exponential growth period and a rapid consumption of the 2 main fermentation products, glycerol and ethanol (figure 3.17c). The plating discrepancy decreased once ethanol and glycerol had been largely consumed but even after 48 h incubation it was still much larger than that normally seen in stationary phase cultures.

### 3.2.3 Respiration versus fermentation

In the aerobic phase of the fermentor batch experiment (figure 3.17) as well as the second exponential growth phase in the 15°C experiment (figure 3.6), *Saccharomyces cerevisiae* was sensitive to a water stress imposed by plating but to a lesser degree than that observed when fermenting glucose. In order to establish whether or not plating hypersensitivity was less when *S. cerevisiae* was respiring rather than fermenting the yeast was grown in a medium that contained a nonfermentable carbon source. A chemically defined medium, Wickerham's medium, was used for these experiments.

For the sake of comparison to *S. cerevisiae*, the stress response of the xerotolerant *S. rouxii* during growth in Wickerham's medium was determined. The viability pattern was essentially the same as that in BYM (figure 3.18, compare with figure...
3.10). The exponential growth rate was about the same in Wickerham's medium and BYM (0.28 generations/h).

When growing exponentially on a nonfermentable carbon source, namely ethanol, *S. cerevisiae* was sensitive to a plating water stress (figure 3.19a). Although ethanol was the main source of carbon (figure 3.19b) it was not the only one. A trace of glycerol, carried over from the inoculum culture, was also present in the culture and was used by the yeast (figure 3.19c). The relatively high concentration of ethanol still found in the medium when the culture was in the stationary growth phase (63 h incubation) indicated that something other than the carbon/energy source was limiting culture growth at this stage of the incubation.

The maximum plating discrepancy for growth on ethanol (2.87) was lower than the maximum plating discrepancy found in the glucose growth cycle (3.69, figure 3.20). The glucose growth cycle plating discrepancy values more closely approximated the ethanol growth cycle values during the second exponential growth phase when ethanol and glycerol were being rapidly utilized by the yeast.

### 3.3 GROWTH RATE AND WATER STRESS PLATING HYPERSENSITIVITY

#### 3.3.1 Batch culture

At any one temperature the magnitude of the plating discrepancy of *Saccharomyces cerevisiae* was correlated not only with the type of carbon metabolism but, on the basis of the glucose and ethanol growth cycles, also the growth rate (table 3.4).

#### 3.3.2 Continuous culture

By growing *S.accharomyces cerevisiae* in continuous culture it was possible to explore the relation between plating discrepancy and growth rate, independent of
FIGURE 3.19a Growth of *Saccharomyces cerevisiae* in Wickerham's medium containing ethanol as the carbon source.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- total count
- MA count
- SHA count
FIGURE 3.19b Concentration of ethanol, the carbon source, in Wickerham's medium during a growth cycle of *Saccharomyces cerevisiae*.

These analyses were performed during the experiment illustrated in figure 3.19a.

FIGURE 3.19c Extracellular glycerol during growth of *Saccharomyces cerevisiae* in Wickerham's medium with ethanol as the carbon source.

These analyses were performed during the experiment illustrated in figure 3.19a.
FIGURE 3.20a Growth of *Saccharomyces cerevisiae* in Wickerham's medium.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- total count
- ▲ MA count
- ■ SHA count

The points represent the mean of two experiments (mean of the log values for total and viable counts).
FIGURE 3.20b Glucose concentration of Wickerham's medium during a growth cycle of *Saccharomyces cerevisiae*.

The points represent the mean of two experiments.

These analyses were performed during the experiment illustrated in figure 3.20a.

FIGURE 3.20c Extracellular glycerol and ethanol during growth of *Saccharomyces cerevisiae* in Wickerham's medium.

- Glycerol
- Ethanol

The points represent the mean of two experiments.

These analyses were performed during the experiment illustrated in figure 3.20a.
Glucose concentration (mM)
carbon and energy source. Glucose was chosen as limiting substrate and was limiting yeast growth in the continuous culture, shown by the rise in total count, dry mass and percentage of c.f.u. after extra glucose was supplied to the yeast (table 3.5). There was a disparity in the relative increases in dry mass and total count after glucose addition. For instance, at the lower dilution rate the total count had approximately doubled 11h after glucose addition whereas the dry mass had only increased by 15%. Evidently the extra glucose induced the production of daughter cells that had much less mass than their mother cells.

**TABLE 3.4 A comparison between exponential growth rate and plating discrepancy in *Saccharomyces cerevisiae* growing in Wickerham's medium at 30°C.**

These values were calculated from figures 3.19a and 3.20a.

<table>
<thead>
<tr>
<th>CARBON SOURCE</th>
<th>EXPONENTIAL GROWTH RATE (generations/h)</th>
<th>MAXIMUM PLATING DISCREPANCY (log MA count-log SHA count)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose*</td>
<td>0.45</td>
<td>3.69</td>
</tr>
<tr>
<td>ethanol</td>
<td>0.13</td>
<td>2.87</td>
</tr>
</tbody>
</table>

*first exponential growth phase

Beck and von Meyenburg (1968) were the first to show that the growth of *S. cerevisiae* in continuous culture under glucose limitation is divided into two phases. Below a critical specific growth rate of about 0.24h⁻¹, the metabolism is completely oxidative while above this critical specific growth rate the metabolism becomes increasingly fermentative. With this change in metabolism increasing amounts of ethanol are found in the growth medium.
TABLE 3.5 Change in dry mass, total count and % budded c.f.u. after glucose addition to steady state cultures of *Saccharomyces cerevisiae*.

<table>
<thead>
<tr>
<th>TIME SINCE GLUCOSE ADDED* (h)</th>
<th>Budded cells (%</th>
<th>Total count (log c.f.u.)</th>
<th>Dry mass (mg/ml)</th>
<th>Budded cells (%</th>
<th>Total count (log c.f.u.)</th>
<th>Dry mass (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady state value</td>
<td>21.9</td>
<td>8.09</td>
<td>3.67</td>
<td>54.4</td>
<td>8.17</td>
<td>3.17</td>
</tr>
<tr>
<td>1.5</td>
<td>36.6</td>
<td>8.20</td>
<td>3.36</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.0</td>
<td>53.0</td>
<td>8.18</td>
<td>3.70</td>
<td>79.8</td>
<td>8.27</td>
<td>3.86</td>
</tr>
<tr>
<td>5.0</td>
<td>37.1</td>
<td>8.33</td>
<td>3.90</td>
<td>46.9</td>
<td>8.32</td>
<td>3.66</td>
</tr>
<tr>
<td>11.0</td>
<td>8.2</td>
<td>8.37</td>
<td>4.20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* 2.5 g of glucose was added as a 50% mass/vol. solution at zero time.

The relation between the size of the plating discrepancy and the growth rate was studied independently of the type of carbon metabolism by selecting dilution rates at which yeast growth was due purely to the oxidative catabolism of glucose. At the fastest dilution rate used, 0.232 h\(^{-1}\) glucose was still limiting growth as indicated by the absence of detectable glucose in the fermentor (table 3.6). The presence of glycerol and ethanol (table 3.6) demonstrated that at least some fermentation of glucose was occurring at this dilution rate but the proportion metabolized this way was probably small judging from the low yields of ethanol and glycerol.
FIGURE 3.21 Growth of *Saccharomyces cerevisiae* in continuous culture.

BYM was used in these experiments.

Top panel: proportion of total c.f.u. that are

- phase-dark
- budded

Bottom panel:

- total count
- MA count
- SHA count

The points represent the mean (mean of the log values for total and viable counts) of six (0.055 and 0.232 h\(^{-1}\)) or twelve determinations (0.027, 0.048, and 0.116 h\(^{-1}\)) and the vertical bars denote the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.
TABLE 3.6 Steady state values at two dilution rates in the continuous cultivation of *Saccharomyces cerevisiae*.

Averages and standard deviations were calculated from six determinations.

<table>
<thead>
<tr>
<th>STEADY STATE VALUES</th>
<th>DILUTION RATE</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.055 h(^{-1})</td>
<td>0.232 h(^{-1})</td>
<td></td>
</tr>
<tr>
<td>Glucose concentration (mM)</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td></td>
</tr>
<tr>
<td>Ethanol (µmol/mg dry yeast)</td>
<td>&lt;0.001*</td>
<td>0.511 ± 0.023</td>
<td></td>
</tr>
<tr>
<td>Extracellular glycerol (µmol/mg dry yeast)</td>
<td>0.018 ± 0.001</td>
<td>0.038 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Cell yield (mg dry yeast/ml)</td>
<td>3.67 ± 0.04</td>
<td>3.17 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

* less than the lowest concentration detectable.

The relation between plating discrepancy and growth rate is illustrated in figure 3.21. The most dramatic change in plating discrepancy was evident between 0.027 h\(^{-1}\) and 0.048 h\(^{-1}\) dilution rates. The other two variables measured that were different at these two dilution rates were the percentage of phase-dark c.f.u. and the MA viability. The phase-dark count was a measure of the number of c.f.u. that were actively growing (Bugeja et al. 1982), phase-dark c.f.u. being equated with growing yeast. The lower phase-dark count at 0.027 h\(^{-1}\) highlighted that the amount of glucose available to the yeast was so low that the majority of cells were not dividing. The total count was the same at 0.027 h\(^{-1}\) and 0.048 h\(^{-1}\) but the number of c.f.u. able to recommence growing on MA was about 40% lower at 0.027 h\(^{-1}\).
3.4 COMPATIBLE SOLUTES AND WATER STRESS PLATING HYPERSENSITIVITY

3.4.1 Polyols

It was mentioned in section 1.2.2.2 that one of the major differences between xerotolerant and nonxerotolerant yeasts is their intracellular composition in high water activity media. Polyols are found in significant quantities in xerotolerant yeasts but this is not the case for the nonxerotolerant species. The significance of intracellular polyol content in water stress plating hypersensitivity was investigated by semi-quantitative analysis of polyols in *Saccharomyces cerevisiae* and the other yeasts previously screened for water stress plating hypersensitivity.

Four polyols were tentatively identified as being present in exponentially-growing yeast (table 3.7). During mid-exponential growth the three hypersensitive species contained either no polyol (*S. cerevisiae*) or trace amounts of glycerol (*C. krusei* and *Kloeckera*). In contrast, species that did not display plating hypersensitivity contained easily detectable amounts of polyols.

An explanation of the resistance of stationary phase or slow-growing cultures was not found in the accumulation of a polyol in the yeast, however. The glycerol content throughout the growth cycle of *S. cerevisiae* is illustrated in figure 3.22; there was no correlation between plating hypersensitivity and glycerol content.

3.4.2 Trehalose in *Saccharomyces cerevisiae*

The possibility that trehalose content affected the viability of *S. cerevisiae* on low water activity agars was investigated because it is found in significant quantities in resting yeast (section 1.2.2.5). In addition, it is an osmoregulatory solute in various bacteria and possibly in yeast as well (section 1.3).
FIGURE 3.22 Glycerol content of *Saccharomyces cerevisiae* during growth in Wickerham's medium.

The points represent the mean of two experiments.

These analyses were performed during the experiment illustrated in figure 3.20a.

FIGURE 3.23 Trehalose content of *Saccharomyces cerevisiae* during growth in Wickerham's medium.

The points represent the mean of two experiments.

These analyses were performed during the experiment illustrated in figure 3.20a.
Glycerol content (μmol/mg dry yeast)
### TABLE 3.7 Intracellular polyol patterns of selected yeasts harvested in mid-exponential growth phase.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>HYPERSENSITIVE</th>
<th>POLYOL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glycerol</td>
</tr>
<tr>
<td><strong>C. albicans</strong></td>
<td>no</td>
<td>++</td>
</tr>
<tr>
<td><strong>C. glabrata</strong></td>
<td>no</td>
<td>+</td>
</tr>
<tr>
<td><strong>C. krusei</strong></td>
<td>yes</td>
<td>+</td>
</tr>
<tr>
<td><strong>C. parapsilosis</strong></td>
<td>no</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Kloeckera</strong></td>
<td>yes</td>
<td>+</td>
</tr>
<tr>
<td><strong>P. miso</strong></td>
<td>no</td>
<td>++</td>
</tr>
<tr>
<td><strong>P. membranaefaciens</strong></td>
<td>no</td>
<td>++</td>
</tr>
<tr>
<td><strong>S. baillii</strong></td>
<td>no</td>
<td>+++</td>
</tr>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td>yes</td>
<td>0</td>
</tr>
<tr>
<td><strong>S. cerevisiae var. ellipsodeus</strong></td>
<td>yes</td>
<td>0</td>
</tr>
<tr>
<td><strong>S. carlsbergensis</strong></td>
<td>no</td>
<td>+</td>
</tr>
<tr>
<td><strong>S. rouxii</strong></td>
<td>no</td>
<td>++</td>
</tr>
</tbody>
</table>

Polyol scoring system:

+ = smallest amount visibly detectable with periodate-Schiff's base reagent on a TLC plate (for glycerol: > 0.02 μmol/mg dry yeast). Increased amounts are denoted by additional + with +++ being the highest possible score.

0 = no polyol detected.

#### 3.4.2.1 Extraction of trehalose

Trehalose is normally extracted from yeast using 0.5M trichloroacetic acid. It was convenient, however, to extract trehalose with ethanol (section 2.6.3) because the other intracellular component measured, glycerol, was routinely extracted with ethanol. No other anthrone-positive material was detectable in ethanol extracts (as determined by TLC using anthrone reagent as the detection spray) and the amount of anthrone-positive material (trehalose) was found to be equal in samples extracted with either ethanol or trichloroacetic acid.
FIGURE 3.24 The relation between plating discrepancy and trehalose content during growth of *Saccharomyces cerevisiae* in Wickerham's medium.

This figure has been compiled from the results presented in figures 3.20 and 3.23.
Plating discrepancy (log MA count-log SHA count)
3.4.2.2 Correlation between trehalose content and plating discrepancy

The trehalose content was measured throughout a growth cycle of *Saccharomyces cerevisiae* growing in Wickerham's medium with glucose as carbon source (figure 3.23). Periods of low trehalose content coincided with periods of large plating discrepancy and, conversely, high trehalose content with small plating discrepancies (figure 3.24).

The intracellular glycerol pattern bore no relation with plating hypersensitivity when *S. cerevisiae* was grown in ethanol medium (figure 3.25). However the relation between trehalose content and water stress plating hypersensitivity (figures 3.26 and 3.27) was found to be broadly similar to that obtained with growth on glucose.

**TABLE 3.8 Trehalose content and plating discrepancy in *Saccharomyces cerevisiae* exposed to oxygen after growing anaerobically for 24 hours in BYM.**

Trehalose values and standard deviations were averaged from two experiments (four estimations) while discrepancy values were averaged from three experiments.

<table>
<thead>
<tr>
<th>Incubation time in aerobic conditions (h)</th>
<th>Plating Discrepancy (log MA count - log SHA count)</th>
<th>Trehalose content (μmol/mg dry yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.55</td>
<td>0.043 ± 0.007</td>
</tr>
<tr>
<td>3</td>
<td>2.89</td>
<td>0.032 ± 0.015</td>
</tr>
<tr>
<td>6</td>
<td>3.40</td>
<td>0.023 ± 0.006</td>
</tr>
<tr>
<td>12</td>
<td>3.34</td>
<td>0.022 ± 0.010</td>
</tr>
<tr>
<td>24</td>
<td>2.81</td>
<td>0.028 ± 0.005</td>
</tr>
</tbody>
</table>
FIGURE 3.25 Glycerol content of *Saccharomyces cerevisiae* during growth in Wickerham's medium with ethanol as the carbon source.

These analyses were performed during the experiment illustrated in figure 3.19.

FIGURE 3.26 Trehalose content of *Saccharomyces cerevisiae* during growth in Wickerham's medium with ethanol as the carbon source.

These analyses were performed during the experiment illustrated in figure 3.19.
Incubation time (h) vs. Trehalose (µmol/mg dry yeast)
Glycerol content (μmol/mg dry yeast)
After 24 h growth in anaerobic conditions the trehalose content of S. cerevisiae was low and decreased further when air was supplied to the fermentor (table 3.8). This decline in trehalose content coincided with a period of exponential growth associated with the utilization of ethanol and an increase in the plating discrepancy (see figure 3.17).

Table 3.9 lists the trehalose and glycerol content of yeast growing at two of the dilution rates described in figure 3.21 and table 3.6. As with the batch experiments the size of the plating discrepancy was correlated with trehalose, but not glycerol, content.

**TABLE 3.9** Plating discrepancy and glycerol and trehalose content in steady state continuous cultures of *Saccharomyces cerevisiae*.

Averages and standard deviations were calculated from six determinations.

<table>
<thead>
<tr>
<th>STEADY STATE VALUES</th>
<th>DILUTION RATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.055 h⁻¹</td>
</tr>
<tr>
<td>Intracellular glycerol (µmol/mg dry yeast)</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>Trehalose (µmol/mg dry yeast)</td>
<td>0.068 ± 0.012</td>
</tr>
<tr>
<td>Plating discrepancy (log MA count-log SHA count)</td>
<td>2.79</td>
</tr>
</tbody>
</table>

There was good agreement between plating discrepancy and trehalose content of *S. cerevisiae* over a wide range of growth conditions (figure 3.28). Much of the scatter
FIGURE 3.27 The relation between plating discrepancy and trehalose content during growth of *Saccharomyces cerevisiae* in Wickerham's medium with ethanol as the carbon source.

This figure has been compiled from the results presented in figures 3.19 and 3.26.
Trehalose (μmol/mg dry yeast)
FIGURE 3.28 The relation between plating discrepancy and trehalose content in *Saccharomyces cerevisiae*.

- Wickerham's medium, glucose as carbon source
- Wickerham's medium, ethanol as carbon source
- BYM, continuous culture
- BYM, aerobic phase of growth in fermentor

A line of best fit has been calculated for the points and is drawn on the figure.

This figure has been compiled from the results presented in figures 3.24 and 3.27 and tables 3.8 and 3.9.
Trehalose (μmol/mg dry yeast)

Plating discrepancy (log MA count-log SHA count)
in the points at the high plating discrepancies was caused by the large standard errors associated with the measurement of SHA viability (see, for example, figure 3.17a). It can be concluded from figure 3.28 that *S. cerevisiae* cells containing more than approximately 0.15 μmole of trehalose (per dry mass) were able to adapt and grow on SHA agar.

### 3.4.2.3 Maltose fermentation and trehalose content

When grown in media with maltose as the carbon source *Saccharomyces cerevisiae* has been reported to contain higher amounts of trehalose during the exponential growth phase than when it grows on glucose (Panek et al. 1979). Water stress plating hypersensitivity was evident, however, when maltose was used as the carbon source (figure 3.29a). Ethanol was the main fermentation product (figure 3.29b) although a small amount of glycerol was also produced (figure 3.29c). The trehalose content (figure 3.29e) at the end of the first exponential growth phase (8 h) was twice that at the equivalent time when glucose was the carbon source. The slower exponential growth rate on maltose (0.040 generations/h) and the correspondingly higher trehalose content was in agreement with the relation between growth rate and trehalose content demonstrated in section 3.3.

In accordance with similar experiments on glucose and ethanol, glycerol content in maltose-grown yeast had no correlation with plating discrepancy (figure 3.29d) while trehalose content did (figure 3.29f). It is apparent from a comparison of figures 3.27 and 3.28 and figure 3.29f that the trehalose 'threshold' value is lower in maltose grown yeast than in ethanol or glucose grown yeast (0.10 μmoles compared to 0.15 μmoles of trehalose per dry mass, respectively).

### 3.4.2.4 Induction of trehalose breakdown in resting yeast

Trehalose breakdown in *Saccharomyces cerevisiae* is via the hydrolytic enzyme, trehalase. The membrane-depolarizing agent 2,4-dinitrophenol is known to cause
FIGURE 3.29a Growth of *Saccharomyces cerevisiae* in Wickerham's medium containing maltose as the carbon source.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- total count
- MA count
- SHA count

The points represent the mean of two experiments (mean of the log values for total and viable counts).
Budded c.f.u. (%) vs. Incubation time (h)

Log10 (count/ml) vs. Incubation time (h)
FIGURE 3.29b Extracellular ethanol during growth of *Saccharomyces cerevisiae* in Wickerham’s medium with maltose as the carbon source.

These analyses were performed during the experiment illustrated in figure 3.29a.

FIGURE 3.29c Extracellular glycerol during growth of *Saccharomyces cerevisiae* in Wickerham’s medium with maltose as the carbon source.

These analyses were performed during the experiment illustrated in figure 3.29a.
FIGURE 3.29d Glycerol content of *Saccharomyces cerevisiae* during growth in Wickerham's medium with maltose as the carbon source.

These analyses were performed during the experiment illustrated in figure 3.29a.

FIGURE 3.29e Trehalose content of *Saccharomyces cerevisiae* during growth in Wickerham's medium with maltose as the carbon source.

These analyses were performed during the experiment illustrated in figure 3.29a.
<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Trehalose (μmol/mg dry yeast)</th>
<th>Glycerol content (μmol/mg dry yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>0.06</td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td>0.09</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>0.12</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td>0.15</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.30</td>
</tr>
</tbody>
</table>

Graphs show the relationship between incubation time and trehalose and glycerol content in dry yeast.
FIGURE 3.29f The relation between plating discrepancy and trehalose content during growth of *Saccharomyces cerevisiae* in Wickerham's medium with maltose as the carbon source.

This figure has been compiled from the results presented in figures 3.29a and 3.29e.
Plating discrepancy (log MA count-log SHA count)

Trehalose (μmol/mg dry yeast)
FIGURE 3.30a  The effect of 2mM 2,4-dinitrophenol on the plate counts of resting *Saccharomyces cerevisiae*.

The experiment was conducted in Wickerham's medium containing no carbon source.

Control culture:  
- MA count
- SHA count

2,4-dinitrophenol culture:  
- MA count
- SHA count

The points represent the mean of the log values from two experiments.

FIGURE 3.30b  The effect of 2mM 2,4-dinitrophenol on the trehalose content of resting *Saccharomyces cerevisiae*.

The experiment was conducted in Wickerham's medium containing no carbon source.

- control culture

- 2,4-dinitrophenol culture

The points represent the mean of two experiments.

These analyses were performed during the experiment illustrated in figure 3.30a.
trehalose breakdown in resting yeast (Berke and Rothstein, 1957) because it activates trehalase (Thevelein, 1984a). Addition of 2,4-dinitrophenol to stationary phase \textit{S. cerevisiae} caused a gradual decline in viability on SHA over a period of 24 h without any corresponding loss in MA viability (figure 3.30a). The trehalose content also decreased over this period (figure 3.30b). During the period of most rapid trehalose breakdown (the first 4 h) there was not, however, a marked loss in the SHA count.

### 3.4.2.5 The \textit{glc1} mutation

An abnormally high level of trehalase activity is found in yeast mutants harboring the \textit{glc1} gene (Ortiz et al. 1983). Because of this high trehalase activity \textit{glc1} mutants are unable to accumulate trehalose (Panek et al. 1978).

The effect of a mutation at the \textit{GLC1} locus on plating hypersensitivity is illustrated in figure 3.31 for the 212-244-1A strain (\textit{glc1}). The low initial SHA count was similar to the value obtained in the mid-exponential phase salt transfer of strain Y41. Whereas a 24 hour old BYM inoculum of strain Y41 was in stationary phase, the higher initial SHA viable count obtained with a 48 hour inoculum (figure 3.32) demonstrated that the 212-244-1A strain needed a longer incubation time to reach a higher level or resistance to the plating stress.

In Wickerham's medium the pattern of glucose consumption (figure 3.33b) and ethanol and glycerol production and utilization (figure 3.33c) was similar in the 212-244-1A strain and in strain Y41, with two exceptions. The 212-244-1A strain produced more glycerol. It also utilized the fermentation products at a slower rate (the growth rate of the 212-244-1A strain in the second exponential growth phase was 0.034 generations/h, approximately half the rate of strain Y41).
FIGURE 3.31 Growth of *Saccharomyces cerevisiae*, strain 212-244-1A (*glc1*), in BYM initiated by a 24 hour old inoculum.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:

- total count
- MA count
- SHA count

The preinoculum and inoculum cultures were grown for 24 hours.

The experimental culture was initiated by a 2.5% inoculum.
FIGURE 3.32 Growth of *Saccharomyces cerevisiae*, strain 212-244-1A (*gld*), in BYM initiated by a 48 hour old inoculum.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:

- total count
- MA count
- SHA count

The preinoculum and inoculum cultures were grown for 48 hours.
The experimental culture was initiated by a 5% inoculum.
FIGURE 3.33a Growth of *Saccharomyces cerevisiae*, strain 212-244-1A (*g*l*c1), in Wickerham's medium.

Top panel: proportion of total c.f.u. that are budded

Bottom panel:
- total count
- MA count
- SHA count

The points represent the mean of three experiments (mean of the log values for total and viable counts) and the vertical bars denote the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.

The preinoculum and inoculum cultures were grown for 48 hours. The experimental culture was initiated by a 5% inoculum.
The low viability on SHA throughout the growth cycle (figure 3.33a) coincided with the low trehalose content of the yeast (figure 3.33e). Like 'normal' yeast (strain Y41), the trehalose content of the 212-244-1A strain decreased during the first exponential growth phase and was followed by a period of trehalose formation. The final trehalose content in the 212-244-1A strain after 48 h growth was approximately 25% of that found in normal yeast.

In addition to the slower second exponential growth rate and low levels of trehalose, the 212-244-1A strain was distinct from strain Y41 in its glycerol content (figure 3.33d), the 18 hour peak being characteristic of the 212-244-1A strain. The 18 hour peak was a reflection of the slower growth rate and higher glycerol production of the 212-244-1A strain. However in the second exponential growth phase, the ratio of the amount of intracellular to extracellular glycerol (1:40) was the same in both strains.
FIGURE 3.33b Glucose concentration of Wickerham's medium during a growth cycle of *Saccharomyces cerevisiae*, strain 212-244-1A (*glc1*).

The points represent the mean of three experiments and the vertical bars denote the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.

These analyses were performed during the experiment illustrated in figure 3.33a.

FIGURE 3.33c Extracellular glycerol and ethanol during growth of *Saccharomyces cerevisiae*, strain 212-244-1A (*glc1*), in Wickerham's medium.

- ■ Ethanol
- ● Glycerol

The points represent the mean of three experiments and the vertical bars denote the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.

These analyses were performed during the experiment illustrated in figure 3.33a.
FIGURE 3.33d Glycerol content of *Saccharomyces cerevisiae*, strain 212-244-1A (*glc1*), during growth in Wickerham’s medium.

The points represent the mean of three experiments and the vertical bars denote the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.

These analyses were performed during the experiment illustrated in figure 3.33a.

FIGURE 3.33e Trehalose content of *Saccharomyces cerevisiae*, strain 212-244-1A (*glc1*), during growth in Wickerham’s medium.

The points represent the mean of three experiments and the vertical bars denote the standard errors of the means. The standard error lay within the symbol if the error bars are not shown.

These analyses were performed during the experiment illustrated in figure 3.33a.
CHAPTER FOUR: DISCUSSION
4.1 THE PHENOMENON OF WATER STRESS PLATING HYPERSENSITIVITY

4.1.1 Interpretation of viability measurements

In a previous study of the physiological response of *Saccharomyces cerevisiae* to a water stress from this laboratory, Edgley and Brown (1983) transferred exponentially growing yeast to salt broth. They measured the viability changes during adaptation to the broth on two agars, one having a high (MA), the other having a low (SHA) water activity. A similar procedure was used in the early stages of this project. The adaptation process was divided into two stages. The 1000 fold drop in MA viability that characterizes the first adaptation stage (figure 3.1) may be the result of dilution stress imposed by plating onto MA or may reflect a genuine decline in the total viability of the liquid culture. Edgley and Brown (1983) obtained no firm evidence for either of these interpretations.

A question arose concerning the recovery at the end of stage 1, as to whether the small number of yeast cells viable at the end of stage 1 are the only ones which multiply in stage 2 or whether the population as a whole recovers and multiplies in stage 2. With the aid of direct microscopic counts of budded and phase-dark c.f.u., this question largely was answered (figure 3.1). Before being transferred to salt broth the budded and phase-dark counts were quite high which is normal for a growing population. After a few minutes in salt broth these counts dropped dramatically and remained low for at least the first 30 h of the adaptation. By the end of stage 1 budded and phase-dark counts had recovered to pre-transfer levels. As these counts are expressed as a proportion of the total count, and the total count did not change throughout stage 1, the recovery in the phase-dark and budded counts indicate that the whole population was recovering from the effects of the salt stress. Therefore the increase in viable counts in stage 2 described by Edgley and Brown (1983) can be attributed to the recommencement of growth of the population as a
whole, not just the growth of the small fraction that was viable on MA or SHA at the end of stage 1.

The simplicity of the *S. cerevisiae* viability pattern in the stationary phase salt transfer experiment (figure 3.2) made interpretation of this experiment easier than the exponential phase transfer experiment. High budded and phase-dark counts preceded the recommencement of growth in salt broth and viability was unaffected by the transfer. Such a viability pattern is similar to that obtained when exponentially growing *S. rouxii* is transferred to 10% NaCl broth (Edgley and Brown 1983). However *S. cerevisiae* was still in lag phase 36 hours after transfer while the *S. rouxii* population has doubled by this time.

The salt transfer experiments used by Edgley and Brown (1983) complicated interpretation of the adaptation sequence as the yeast was subjected to two water stresses, firstly when transferred to the salt broth and later when plated onto the malt agar. To eliminate the second water stress and to give a clear idea of what happens simply during a growth cycle, yeast was exposed to a water stress by plating onto low water activity agar during a growth cycle in normal high water activity medium. This experimental method had an advantage as the imposed stress also permitted the yeast's response to be quantified. This experiment led to the discovery of the phenomenon that has been termed "water stress plating hypersensitivity". The term describes that physiological state of *S. cerevisiae* in which less than $10^{-3}$ of the population survives when plated onto a low water activity agar. Typically a *S. cerevisiae* culture growing exponentially displays plating hypersensitivity.

Low water activity is a selective force in nature (Yancey et al. 1982) so it is possible that plating *S. cerevisiae* onto low water activity agar selected variants that were inherently more resistant to a water stress during exponential growth.
If this were true, a colony found on low water activity agar during the mid-exponential growth phase should, upon cultivation in high water activity liquid medium and subsequent plating onto low water activity agar, give viable counts approximately equal to MA counts during exponential growth. This, in fact, was not the case (Mackenzie et al. 1986). Therefore the ability of a small proportion of cells to produce colonies on low water activity agars was not due to the genetic heterogeneity of the population.

It could be argued that the failure to grow on low water activity agars is attributable to the cells entering a state of dormancy after being plated onto these agars. A yeast cell has three options if it is in a dormant state. It may die, start growing again or remain dormant. Mackenzie et al. (1986) tested whether S. cerevisiae entered a dormant state after exposure to low water activity agar. They concluded that cells present but not visible as colonies on low water activity agar at the time of colony counting were dead.

4.1.2 Physiological characteristics
The lethal effects of low water availability on growth of exponential phase Saccharomyces cerevisiae on agar was not evident until the water activity of the agar was below a threshold value. In the case of salt agar there was no discernible effect on viable count when the salt content of the agar was less than 5% (figure 3.8), while 30% glucose is the threshold value for SHA agar (Mackenzie et al. 1986). The water activities of the 5% salt and 30% sugar agars are 0.972 $a_w$ and 0.962 $a_w$, respectively (calculated from Robinson and Stokes 1965). Together with evidence from other experiments in this thesis, in particular the S. cerevisiae growth cycle where viability was determined on salt and glucose agars (figure 3.3), it is clear that for a given water activity, NaCl is more inhibitory than glucose. Tolerance of high concentrations of a specific solute, as opposed to low water activities in general, has been reported for numerous yeasts. For instance,
Debaryomyces hansenii tolerates high NaCl concentrations (Norkrans 1966) while there are strains of S. rouxii which are salt (NaCl) but not sugar (sucrose) tolerant and others which are sugar but not salt tolerant (Onishi 1963; Mori and Windisch 1982).

Supplementation of salt agar with glycine betaine or glycerol is known to improve tolerance of low water activity in some microorganisms. Glycine betaine is a compound that does not inhibit enzyme function at relatively high concentrations (Pollard and Wyn Jones 1979). It plays a major role in osmoregulation in the extremely halophilic phototrophic bacterium, Ectothiorhodospira halochloris (Galinski and Truper 1982), in the moderately halophilic bacterium Ba1 (Rafaeli-Eshkol and Avi-Dor 1968; Shkedy-Vinkler and Avi-Dor 1975), in some halophilic eubacteria (Imhoff and Rodriguez-Valera 1984), several members of the Enterobacteriaceae (Le Rudulier and Bouillard 1983; Le Rudulier et al. 1984; Perroud and Le Rudulier 1985) and several strains of Rhizobium (Sauvage et al. 1983; Le Rudulier and Bernard 1986). When glycine betaine is supplied to these microorganisms growth at high salt concentrations is stimulated. However it did not improve the viability of S. cerevisiae during the hypersensitive period. It is noteworthy that only the more salt tolerant strains of Rhizobium, including R. meliloti, show improved growth in salt medium after the addition of glycine betaine. R. meliloti can metabolize glycine betaine at high but not at low water activities. Instead the betaine is accumulated against a large concentration gradient at low water activities (Le Rudulier and Bernard 1986). Regulation of the transport systems responsible for the uptake of glycine betaine have been extensively studied in the enteric bacteria. It is not possible to say whether the absence of a glycine betaine uptake system or some other factor(s) was the reason why S. cerevisiae showed no improvement in viability on glycine betaine salt agar during the hypersensitive period.
Two reports have documented improved growth of yeast at low water activities upon addition of glycerol to the growth medium. In a mutant strain of *Debaryomyces hansenii* with a decreased ability to retain glycerol and arabitol at high salinities, the length of the lag phase and generation time in NaCl medium is reduced if the medium is supplemented with glycerol (Adler and Gustafsson 1980). Glycerol contributes to the maintenance of osmotic balance and, as less energy is expended on polyol production and accumulation, the mutant is able to grow more rapidly. Anand (1969) observed that a sugar-tolerant strain unable to grow at 0.85aw (in medium adjusted with sucrose) could do so when the water activity is lowered further to 0.80 aw by the addition of glycerol. The underlying mechanism for the improved xerotolerance of this strain is not known. Presumably, as for the *D. hansenii* mutant, this yeast uses the supplied glycerol to maintain polyol levels compatible with growth.

Of some relevance to the question why *S. cerevisiae* showed no improvement in viability on salt agar supplemented with glycerol (figure 3.7), is one of the known differences between xerotolerant and nonxerotolerant yeasts. In the xerotolerant *D. hansenii* (Gustafsson and Norkrans 1976) and *S. rouxii* (Brown 1978a; Edgley and Brown 1978), intracellular glycerol increases as environmental water potential is lowered. These yeasts can maintain very large concentration gradients across their cytoplasmic membranes by virtue of an active transport system for glycerol (Adler et al. 1985; Brown 1974). The glycerol content of *S. cerevisiae* also increases as environmental water potential is lowered. However, this is achieved by producing more glycerol, not by establishing a large concentration gradient across the membrane (Brown 1978a; Edgley and Brown 1978); *S. cerevisiae* does not have an active transport mechanism for glycerol (Brown 1974). So the yeast's viability on salt agar was not improved when supplied with glycerol presumably because it could not use the glycerol as an osmoregulatory solute.
Incubation of *S. cerevisiae* in a 24% glucose solution prior to plating eliminated plating hypersensitivity (figure 3.9). In a similar experiment Mackenzie et al. (1986) found that incubating exponentially growing *S. cerevisiae* in a 2% NaCl solution for about one hour before plating also improved the yeast's resistance to low $a_w$ agar. The underlying reason for the acquired resistance was suggested by Mackenzie et al. (1986) to be due to the accumulation of high levels of intracellular glycerol during the incubation period. They observed that plating hypersensitivity is eliminated if *S. cerevisiae* is grown in medium containing at least 2% NaCl. However if washed prior to plating, a process known to remove intracellular glycerol (Edgley 1980), the suspension becomes sensitive. It seems that the amount of glycerol (approximately 2 μmole/mg dry yeast) accumulated during growth in the NaCl medium is sufficient to allow *S. cerevisiae* to overcome the lethal effects of plating onto SHA.

This thesis has documented a differential growth phase sensitivity of *S. cerevisiae* to a water stress. The occurrence of exponential phase sensitivity to a water stress was tested in 12 yeast species from 5 genera (table 3.3). One of these yeasts, *S. rouxii*, is well known for its xerotolerance and has been reported to grow in high sugar media having water activities as low as 0.62 $a_w$ (see, for example Brown 1976). The viability of this yeast on MA and SHA was almost identical in all growth phases of batch culture (figure 3.10 and figure 3.18). None of the other xerotolerant yeasts tested, including *D. hansenii* (Mackenzie et al. 1986) were sensitive either. Furthermore some nonxerotolerant yeasts (table 3.2) did not have a hypersensitive period; notable among these was *S. carlbergensis*. A major distinction between the resistant species and the closely related sensitive strains was the presence of more than one polyol in the former but not the latter species during the exponential growth phase (table 3.7). The significance of intracellular
polyols during the exponential growth phase to tolerance of low water activity agar will be discussed in section 4.2.

In the present context it is interesting to note that Spencer (1968) observed low ethanol yields in xerotolerant yeasts growing in glucose medium. This suggests that the xerotolerant species were resistant to an exponential phase water stress because, under the conditions used to screen for water stress plating hypersensitivity (aerated, glucose medium), they largely catabolized glucose respiratively. Conversely, S. cerevisiae utilized glucose respiro-fermentatively (Fiechter et al. 1981; Kappeli 1987) in aerated glucose medium as evidenced by the high amount of ethanol produced (figure 3.15b). However, conditions have been identified where S. cerevisiae does utilize glucose respiratively with low yields of ethanol, making it possible to assess the relation between water stress resistance and respiratory glucose catabolism.

Continuous culture studies have shown that the respiratory breakdown of glucose without ethanol formation is possible in S. cerevisiae when it is grown at low dilution rates in a glucose-limited chemostat (Beck and von Meyenburg 1968; Rieger et al. 1983). The response of the yeast to a water stress in a glucose-limited continuous culture is illustrated in figure 3.21. Analyses of the medium demonstrated ethanol was not being produced in detectable amounts at the 0.055h⁻¹ dilution rate (Table 3.6), indicating that respiratory glucose breakdown was occurring at this dilution rate. Yet the plating discrepancy was high. Clearly, respiratory catabolism of glucose does not confer water stress resistance to S. cerevisiae and other factors besides this one must account for the resistance of the xerotolerant yeasts to an exponential phase water stress.
4.2 COMPATIBLE SOLUTES AND OSMOREGULATION

4.2.1 Exponential phase resistance to a water stress

Resistance of mid-exponential phase yeast to plating on a low water activity growth medium was, without exception, associated with accumulation of polyols to a significant level. Resistant yeasts contained a minimum of 0.02 μmole/ mg dry yeast glycerol and at least one other polyol in equal or greater amounts (table 3.7). In contrast, none of the sensitive yeasts contained more than one polyol in detectable quantities and, in the case of the hypersensitive *Saccharomyces cerevisiae*, none was detected. Polyols were not totally absent from *S. cerevisiae*. In both Wickerham's medium (figure 3.22) and BYM (Edgley and Brown 1983) intracellular glycerol was present but it was not detectable by TLC (below 0.02 μmole/ mg dry yeast).

Yeasts producing more than one polyol have been recognized by Onishi (1960a), Spencer (1968), Brown and Simpson (1972) and Spencer and Spencer (1978) as forming a distinct group: the xerotolerant yeasts. Edgley (1980) has argued that, in *S. rouxii*, arabitol and glycerol synthesis serves to regenerate NADP+. The activity of phosphofructokinase is quite low in this yeast (Brown and Edgley 1980); this and other evidence implies that *S. rouxii* relies heavily on the pentose phosphate pathway for the catabolism of glucose. When glucose is used rapidly by the pentose phosphate pathway NADPH will be generated in amounts that presumably exceed the yeast's biosynthetic requirements in most experimental situations. Polyol formation reoxidizes NADPH and should serve to facilitate the continuation of glucose consumption. A similar role for the high polyol production typical of some fungi has also been proposed by Lewis and Smith (1967).

Comparative glycerol production studies at high water activities between the resistant *S. rouxii* and the hypersensitive *S. cerevisiae* illustrates an important distinction between these two yeasts. Brown (1978a) found they differ in the
manner of their retention of glycerol. *S. rouxii* retains much more of the glycerol it produces than does *S. cerevisiae* which suggests these yeasts may also differ in their plasma membrane properties. Edgley (1980) demonstrated a difference in the linoleic acid (C18:2) content of these yeasts. As Walker and Kummerow (1964) reported a correlation between the linoleic acid content of erythrocytes and their permeability to glycerol, the lower glycerol permeability of *S. rouxii* may be due to the high linoleic acid content of its plasma membrane, in contrast to the high glycerol permeability of the plasma membrane of *S. cerevisiae* which has a low linoleic acid content. One further difference between these yeasts which probably contributes to the high retention of glycerol in *S. rouxii* is that glycerol is actively transported in this yeast. In contrast an active glycerol transport system does not exist in *S. cerevisiae* (Brown 1974, 1978a).

### 4.2.2 Stationary phase resistance to a water stress

In common with the xerotolerant *Saccharomyces rouxii* (Brown 1978a) and *Debaryomyces hansenii* (Gustafsson and Norkrans 1976), *S. cerevisiae* contained trace amounts of glycerol in the stationary growth phase in high water activity medium (figure 3.22). While glycerol content is low, arabitol content is at its highest during stationary phase in the two xerotolerant yeasts (Brown 1978a; Gustafsson and Norkrans 1976). A partial explanation for the stationary phase resistance to a water stress in *S. rouxii* and *D. hansenii* is therefore found in the presence of arabitol. However this does not explain the stationary phase resistance of *S. cerevisiae* since no polyol other than glycerol was detected in this yeast (table 3.7).

When *S. cerevisiae* is starved of glucose, such as at the end of the first exponential growth phase in glucose medium, trehalose has been reported to accumulate (Panek 1962; Polakis and Bartley 1966; Grba et al. 1975; Panek 1975; Panek and Mattoon 1977; Quain and Haslam 1979; Lillie and Pringle 1980). According to Lillie and
Pringle (1980) trehalose accumulation does not begin until the external glucose concentration is about 3mM, at which point trehalose content increases from approximately 0.009 μmole/mg dry yeast to about 0.09 μmole/mg dry yeast three hours after glucose exhaustion. Even higher amounts of trehalose have been reported. For example, Panek and Mattoon (1977) found their strain accumulates about 0.24 μmole trehalose /mg dry yeast two hours after glucose is exhausted from the medium. The Y41 strain of *S. cerevisiae* used in this study did not accumulate trehalose at the end of the first exponential growth phase on glucose to the levels reported in the literature (figure 3.23). In the anaerobic growth experiment trehalose content remained low even after 12h had elapsed since glucose exhaustion (figure 3.17b and table 3.8). Trevelyan and Harrison (1956) also documented reduced trehalose accumulation during anaerobic fermentation. The trehalose content of the Y41 strain after the first exponential growth phase in maltose medium was lower than the values Panek et al. (1979) reported. The trehalose accumulation pattern of the Y41 strain in maltose medium seems to be the exception rather than the rule for *S. cerevisiae*.

Quite clearly trehalose is of major importance in determining stress resistance of stationary phase *S. cerevisiae*. Under a variety of conditions the average trehalose content of a yeast suspension correlated with the magnitude of the plating discrepancy (figure 3.28). The values obtained from the glc1 mutant growth cycles are close to or on the line of best fit drawn in figure 3.28. From figure 3.28, a trehalose content of approximately 0.15 μmole/mg dry yeast is sufficient to protect *S. cerevisiae* completely against the stress. When grown with maltose as carbon source the trehalose content required for protection was lower, about 0.10 μmole/mg dry yeast (figure 3.29f). The significance of this lower threshold value is unclear. Yeast containing a trehalose content equal or greater than the threshold level are completely protected against the stress imposed by plating onto SHA in the specified growth conditions but may not be protected against a more severe stress.
Water stress plating hypersensitivity is a binary phenomenon. The viability measurements distinctly divided the population into a group sensitive and a group resistant to the imposed water stress. The trehalose values on the other hand are averaged measurements of the bulk population. No conclusion can be drawn about the distribution of trehalose in the portion of the population that is sensitive to the water stress except that those c.f.u. which do not have a trehalose content equal to the critical value (approximately 0.15 µmole/mg dry yeast) do not tolerate a water stress of this specific magnitude.

It is common for resting microorganisms to be more resistant to physical stresses than when they are multiplying. By using two other experimental approaches it was possible, however, to demonstrate that resistance of S. cerevisiae to a water stress was not invariably associated with the stationary growth phase but always correlated with trehalose content. In the first approach, the breakdown of trehalose in stationary phase yeast without stimulating growth induced stress sensitivity (figure 3.30). However the correlation between SHA viability and trehalose content was not exact in the sense that the period of most rapid trehalose breakdown (first four hours) did not coincide with the period of most rapid loss of SHA viability (between 10 and 15 hours). There is an implication here that something else in involved but what that might be has yet to be identified. Berke and Rothstein (1957) found that most of the trehalose was broken down within the first two hours after addition of 2,4-dinitrophenol, a similar finding to this thesis. 2,4-dinitrophenol causes trehalose breakdown by initiating a cAMP-dependent phosphorylation cascade system which regulates trehalase activity (Van der Plaat and Van Solingen 1974; Van Solingen and Van der Plaat 1975; Chvojka et al. 1981; Ortiz et al.1983). Normally this regulatory system is triggered by the addition of glucose to stationary phase yeast. Glucose is thought to depolarize the yeast membrane because it is cotransported into the yeast with protons (Seaston et al. 1973; Slayman and Slayman 1974). 2,4-dinitrophenol also depolarizes the yeast membrane (Thevelein 1984b) although Valle
et al. (1986) argue that intracellular acidification rather than membrane depolarization triggers the regulatory system that activates trehalase. Depolarization of the membrane (or internal acidification) activates adenylate cyclase (Trevillyan and Pall 1979; Thevelein 1984b) which results in an increase in cAMP concentration, as has been observed by Van der Plaat (1974), Mazon et al. (1982), Tortora et al. (1982), Thevelein (1984c) and Thevelein and Beullens (1985). One or more cAMP-dependent protein kinases are subsequently activated which in turn phosphorylates trehalase. By this phosphorylation, cryptic trehalase is converted into a highly active form which is able to catalyse the breakdown of trehalose. Besides causing trehalose breakdown the addition of 2,4-dinitrophenol to resting yeast is known to induce a number of other changes. These include increases in the cAMP content (Trevillyan and Pall 1979; Mazon et al. 1982), fructose 2,6-bisphosphate content (Francois et al. 1984) and 6-phosphofructo-2-kinase activity (Francois et al. 1984) and a decrease in fructose 1,6-biphosphatase activity (Mazon et al. 1982).

A second experimental approach also demonstrated that resistance of *S. cerevisiae* to a water stress was not invariably associated with the stationary growth phase. Water stress plating hypersensitivity was examined in a *S. cerevisiae* strain defective in trehalose metabolism. As mentioned in section 3.4.2.5, the *glc1* mutation diminishes trehalose accumulation during nonproliferating conditions (Panek et al. 1979; Padrao et al. 1982), trehalose accumulation being prevented because trehalase is present in its phosphorylated active form in *glc1* mutants (Ortiz et al. 1983). This strain neither accumulated trehalose to 'normal' levels in stationary phase nor did it develop resistance to the plating stress (figures 3.32, 3.33a and 3.33e). The correlation between the trehalose content of the 212-244-1A strain and the magnitude of the plating discrepancy (not shown) lay within experimental variation on the regression line in figure 3.28. This agreement together with other experimental evidence discussed leaves little doubt that trehalose content is an important determinant of the water stress response of *S. cerevisiae*. 
Besides the loss of the capacity to accumulate trehalose in non-proliferating conditions, other phenotypic characteristics of mutants harbouring of \textit{glc1} mutants have been described (Padrao et al. 1982). The other major trait is their glycogen deficiency (Rothman-Denes and Cabib 1970). Glycogen, like trehalose, is a storage carbohydrate in yeast and its synthesis is associated with yeast entering the stationary growth phase (Chester 1963; Eaton 1960; Fales 1951; Lillie and Pringle 1980) and the diauxie lag phase (Polakis and Bartley 1966; Gunja-Smith et al. 1977; Quain and Haslam 1979; Lillie and Pringle 1980). In general the patterns of accumulation of glycogen and trehalose are similar during growth in glucose medium. No attempt was made in this study to correlate glycogen content with the magnitude of the plating discrepancy.

Mackenzie et al. (1986) reported that plating hypersensitivity was eliminated when the glycerol content of \textit{S. cerevisiae} was about 2 \textmu mole/mg dry yeast. For trehalose, the threshold value for protection against a stress of identical magnitude was approximately 0.15 \textmu mole/mg dry yeast. So under these conditions trehalose is a better solute than glycerol in protecting \textit{S. cerevisiae} from a sudden water stress.

While trehalose is obviously a very effective compatible solute, it should not be assumed that all protective agents have the same mode of action nor that their mode of action is identical from one organism to the next. While trehalose has been reported to be an osmoregulatory solute in \textit{Escherichia coli} (Ström et al 1986) and many freshwater and brackish cyanobacteria (Mackay et al. 1984; Larsen et al. 1987), the evidence for an osmoregulatory role for the disaccharide in yeast is tenuous (section 1.2.2.5). Trehalose has been found to be the most effective compound out of eleven polyhydroxylic compounds tested in stabilizing the structure of both isolated lipoprotein membranes and lipid monolayers, glycerol was the least effective (Crowe et al 1984). Thus it is conceivable that \textit{S. cerevisiae} is primarily protected at the
plasma membrane level by trehalose with its protective effects perhaps being on processes like ion transport. Glycerol seems to protect enzyme activity (Brown 1978) and is also known to stimulate transcription in both procaryotic (Nakanishi et al. 1974) and eucaryotic systems (Buss and Stalter 1978).

4.2.3 Distinction between osmoregulatory and compatible solutes

In a high water potential environment all the intracellular solutes (intermediary metabolites, proteins, nucleotides and salts) contribute to the maintenance of a turgor pressure and volume compatible with yeast growth. During a growth cycle the relative contributions of glycerol and trehalose fluctuates greatly in *Saccharomyces cerevisiae* when growing in glucose (figures 3.22 and 3.23), ethanol (figures 3.25 and 3.26) and maltose (figures 3.29d and 3.29e). When the yeast is adapted to low water activities, glycerol makes the greatest contribution to the maintenance of turgor pressure in *S. cerevisiae* (Brown 1978a). However it is interesting to note the similarity in the pattern of glycerol content during a growth cycle in 10% NaCl medium (Brown 1978a) and in high water activity medium (figure 3.22). Under both conditions glycerol content was highest in the exponential growth phase and lowest in the stationary phase. Glycerol content during a growth cycle in saline media shows a similar trend in *Debaryomyces hansenii* (Adler and Gustafsson 1980) and *S. rouxii* (Brown 1978a). The other osmotically active substance that fluctuates greatly during the growth cycles of *S. rouxii* and *D. hansenii*, both in high and low water activity media, is arabitol although the overall production of arabitol is not particularly sensitive to water potential (Brown 1978a; Adler and Gustafsson 1980). High intracellular amounts of arabitol are associated with the stationary growth phase, whereas it is rapidly broken down (*D. hansenii*, Adler and Gustafsson 1980) or released (*S. rouxii*, Brown 1974) when the yeasts are supplied with fresh glucose. Both trehalose and arabitol (Lewis and Smith 1967; Nobre and Da Costa 1985) are storage compounds or reserve fuels, interestingly with similar patterns of accumulation. Both substances necessarily contribute to osmotic potential but the
content of each on present evidence is more sensitive to the phase of growth or the status of energy metabolism than it is to water potential.

Trehalose in *S. cerevisiae*, arabitol in *S rouxii* and *D. hansenii*, and the polyols present in the exponential growth phase in the other resistant yeast species represent a third type of solute important in survival of a water stress. These polyols and trehalose are conceivably important in two situations: in maintaining turgor pressure in non-proliferating yeast and in protecting yeast against the potentially lethal effects of a sudden water stress. Until now the term compatible solute has been used to refer to osmoregulatory solutes that are protective of enzyme function. Edgley and Brown (1983) did however recognize that the chemical characteristics of arabitol would enable it to act as a reserve or supplementary compatible solute. The role of trehalose, arabitol and other polyols in preventing water stress plating hypersensitivity is a clear example of solutes acting as protective solutes, helping to maintain intracellular conditions compatible with survival and recovery from a water stress. Their contribution to the maintenance of turgor pressure is probably minor under most conditions with the possible exception of non-proliferating conditions. Thus these compatible solutes form a group of solutes distinctly different from the other osmoregulatory, compatible solutes.
4.3 CONCLUSION

A new phenomenon was discovered during a growth cycle of *Saccharomyces cerevisiae* in conventional high water activity medium. It was called 'water stress plating hypersensitivity'. This phenomenon was characterized by a major difference between counts on high $a_w$ agar medium and those on low $a_w$ or 'stressing' agar medium. Most of the other yeasts tested did not display water stress plating hypersensitivity. The resistant yeasts were protected by virtue of their accumulation of one or more polyols even before they were subjected to a solute stress. The sensitive yeasts had little or no polyol when they were grown in a conventional $a_w$ medium but, when they were 'preconditioned' by exposure to a moderate solute stress, they accumulated glycerol (Mackenzie et al. 1986) and became resistant to the plating stress. Both glycerol and the acquired resistance were lost when such yeasts were washed with water. Resistance to the plating stress is thus conferred by an adequate content of a polyol. Polyols, however, do not explain the resistance developed in stationary phase by *S. cerevisiae* in batch culture or at low growth rates in continuous culture. Under these circumstances resistance was conferred by the accumulation of trehalose in response to changes in the dynamics of carbohydrate metabolism.


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Selected aspects of microbial osmoregulation

(Osmoregulation; compatible solutes; water stress; eukaryotes; cell physiology; homeostasis)

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1. SUMMARY

Some salient characteristics of microbial osmoregulation are reviewed, with specific examples drawn from eukaryotes. As well as the need for an osmoregulatory solute to be 'compatible' with cellular processes under all conditions, the importance of the physiological method of regulating the content of the solute as a factor determining xerotolerance is emphasized. The significance of turgor/volume homeostasis is discussed and examples are cited in which, during exponential growth, there is apparently no homeostatic control of the cellular content of the major osmoregulatory solute. Some implications of this for the overall mechanism of osmoregulation are considered.

A recent experiment is described which raises questions about the timing of an osmoregulatory 'signal' in Saccharomyces cerevisiae. Other experiments are summarized which distinguish between osmoregulatory and compatible solutes in yeast. These experiments implicate trehalose as a non-osmoregulatory compatible solute in certain circumstances.

2. INTRODUCTION

Our task in this review is to give an 'overview' of the phenomenon of osmoregulation in microorganisms. It is a formidable task. Studies in osmoregulation in general can be broadly divided into two distinct areas. One is biophysical, in which attention is focused on direct measurement of physical parameters such as membrane potential and turgor pressure. The second is more descriptive, and is concerned with the biochemistry and broader aspects of the cell physiology of osmoregulation. Microorganisms have generally not been amenable to very precise biophysical studies, at least partly because microbial cells for the most part are too small for relevant physical measurements to be made with a satisfactory degree of precision and accuracy. Recently, however, Walsby [1] has exploited the pressure/volume relations of gas vacuoles to measure directly turgor pressure in cyanobacteria. A more precise approach to the biophysics of microbial osmoregulation might thus be possible in the future, at least in those organisms with gas vacuoles.

Until now, however, most of the relevant work has been at the levels of biochemistry and cell physiology. Since this is also the area of our own experience, our comments will be restricted to that aspect. Moreover, because of space limitations, examples will be confined to eukaryotes although...
principles are equally applicable to prokaryotes.

Specifically, we wish to do 3 things, namely: (1) emphasize the importance of the physiological method of osmoregulation in determining microbial water relations; (2) question some basic assumptions about the physiology of osmoregulation; (3) distinguish between osmoregulatory and compatible solutes.

3. DEFINITION

In a previous review [2] we defined osmoregulation as 'the maintenance of approximately constant cell volume and turgor pressure in the face of changing water potential'. For reasons that should become apparent in due course, we now wish to use a somewhat different working definition, namely the maintenance of turgor pressure and/or cell volume within limits necessary for growth and multiplication of an organism. (Whether or not 'osmoregulation' is itself a satisfactory term is a matter on which we do not wish to comment at this stage. The questions raised by Reed [3] about terminology, however, deserve serious consideration.)

4. METHODS OF OSMOREGULATION

Under appropriate conditions, microorganisms of various kinds can thrive in environments from those that are very close to distilled water (water activity $a_w$ 1.00) down to about $a_w$ 0.62 and at salinities from effectively zero to saturated NaCl (6.2 molal at 30°C, $a_w$ 0.75) [4]. In order to tolerate the lower levels of $a_w$, a microorganism must accumulate a compatible solute. In order to adapt to a change from one water activity to another, however, it must osmoregulate, that is it must control its volume or turgor pressure or both. It does this by regulating the content of one or more osmoregulatory solutes.

At high water activity, the concentration of an osmoregulatory solute is low enough for intermediary metabolites or the inorganic ions that commonly accumulate to fill this role without exerting any generalized inhibition or toxicity. Thus, in a dilute environment, a relatively wide spectrum of osmoregulatory solutes is encountered. As water activity or water potential is lowered, however, the environment becomes progressively more selective of osmoregulatory solutes just as it does of organisms. Under severe conditions, an osmoregulatory solute attains a high intracellular concentration (several molal) and then toxicity is a potential problem. If an organism is to thrive in this situation, an osmoregulatory solute must be 'compatible' with the entire range of essential cellular functions. At extremely low levels of water activity the only osmoregulatory solute so far identified in eukaryotic microorganisms is glycerol [5].

Accumulation of glycerol is not in itself sufficient to ensure growth at very low water activity although it does seem to be an essential prerequisite. For example, the two yeasts *S. cerevisiae* and *Saccharomyces rouxii*, when grown under optimal conditions, will respectively tolerate about $a_w$ 0.90 and 0.62 (each is much less tolerant of a saline environment). Both yeasts osmoregulate with glycerol as do the salt-tolerant yeast *Debaryomyces Hansenii* and the uniquely salt-tolerant unicellular alga *Dunaliella* [5-7].

Brown and Borowitcka [7] have listed some of the more representative methods of controlling the content of an osmoregulatory solute of which the following three are of immediate relevance:

(1) An approximately constant total yield (per cell) of osmoregulator is produced, more or less independently of water activity, but the proportion retained within the cell is controlled by permeation/transport in response to water activity (e.g., glycerol and *S. rouxii*).

(2) The total yield (per cell) of osmoregulator is controlled in response to water activity and a constant proportion of it is retained within the cell (e.g., glycerol and *S. cerevisiae*).

(3) The total yield (per cell) of osmoregulator is controlled in response to salinity (or water activity) and virtually all of it is retained within the cell (e.g., glycerol and *Dunaliella*).

Mechanisms (1) and (2) involve extensive leakage to the medium. Nevertheless, Method (1) is conservative. Method (2) is extremely wasteful and, although it is not the whole explanation, this
wastefulness is a sufficient reason why *S. cerevisiae* is substantially less xerotolerant than *S. rouxii*. Method 3 is very conservative and effective (see also [4,7]).

There are many biochemical characteristics that are obviously related to the differences in methods of glycerol production by the two exemplar yeasts. For example, when grown in 10% (w/v) NaCl, *S. cerevisiae* produces about 40 times as much glycerol-3-phosphate dehydrogenase (NAD-linked) as it does in a conventional high water activity growth medium. No such response is given by *S. rouxii* [8].

In theory there should be two levels at which an organism can regulate its volume or turgor pressure. One is homeostatic, that is the maintenance of turgor or volume within the limits necessary for growth. The other is adjustment to changing water potential or water activity. Perhaps osmoregulation is most refined at the level of homeostasis, inasmuch as the other aspect, the adjustment to change, might simply involve the removal of a control—in some cases. It might even be that, in a strict sense, the homeostatic mechanism is the only valid example of osmoregulation, adaptation perhaps being essentially the removal of a control with its reimposition at the end of the adaptation sequence. It might also be, of course, that in phrasing the question in this way we are merely playing with semantics.

Whether or not this suggestion is valid, however, we wish to assert that osmoregulation as a phenomenon cannot be understood until the homeostatic aspect is understood and, in turn, this cannot be understood in a vacuum. Osmoregulation with metabolites is a special case of general metabolic regulation and can ultimately be interpreted only in that light. Moreover, the situation is complicated because many organic osmoregulatory solutes function also as reserve fuels and probably all can participate in 'other' non-osmoregulatory metabolic sequences.

If an osmoregulatory solute is a metabolite, the ramifications of controlling its content can be quite different from one metabolic sequence to another. For example, the implications of regulating the content of glycerol produced predominantly by glycolysis (*S. cerevisiae*), via the pentose phosphate cycle (*S. rouxii*) or by the short sequences between dihydroxyacetone phosphate and glycerol sometimes called the 'glycerol cycle' (*Dunaliella*) [9] are quite different in each case [8]. Moreover, whether or not there is one unique signal that triggers an osmoregulatory response, to expect a single transduction site of that signal is probably as naive as an expectation of a unique rate-limiting step in a metabolic sequence (for example, see [10]).

Let us now return to the question of homeostasis. Most microbiological experimentation is conducted with batch cultures. In batch culture, no nutrient is at a rate-limiting concentration while the population is growing exponentially but the composition of the growth medium changes progressively during a growth cycle. Nevertheless, the assumption is usually made that, while a population grows exponentially, it is in a steady state and the cells are of constant average chemical composition, biochemical activity and size (with some qualifications about size for budding yeasts). A logical extrapolation from these assumptions is that turgor and hence the concentration of the main osmoregulatory solute are also constant.

These assumptions are demonstrably false, at least in some cases. For example, the salt-tolerant yeast, *D. hansenii* accumulates both arabitol and glycerol. The arabitol content responds slightly to salinity but the major osmoregulator is glycerol. Neither polyol is maintained at a constant intracellular concentration during exponential growth but the actual pattern of accumulation is dependent on the salinity of the medium [6]. In 2.7 M NaCl, for example, the glycerol content ranges between effectively zero and 3 μmol·mg⁻¹ (approx. 2 molal) dry yeast during a growth cycle: during the actual period of exponential growth, the range is approx. 1.6–3.0 μmol·mg⁻¹ dry yeast with a sharp peak in mid-exponential phase. On the other hand, the content of arabitol changes only slightly but progressively during exponential growth and reaches its highest level well within the stationary phase [6]. Its accumulation in no way compensates osmotically for the substantial variation in glycerol content during exponential growth. We also find that intracellular glycerol in *S. cerevisiae* is far from constant during exponential
growth (Singh et al., unpublished results).

These yeasts thus are clearly not biochemically homeostatic during exponential growth. If, in spite of this, they are strictly osmotically homeostatic then their osmoregulatory mechanisms are much more complex and sophisticated than previously suspected. If, on the other hand, they do not compensate at all, then their osmoregulation is much cruder than we have believed and they tolerate substantial fluctuations in turgor pressure during a growth cycle. If the yeasts do have a capacity for strict biochemical homeostasis, it is likely to be recognizable only in continuous culture. Even here, however, there is reason for uncertainty since glycolysis is classically an oscillating system and, in addition, oscillations are common in microbial populations, including those of yeasts, in continuous culture [11].

It should not be difficult to answer all of these questions with more comprehensive analyses and there are good reasons why this should be done. For the present, however, we can only guess at what happens. Since, in the example quoted for \textit{D. hansenii}, glycerol was well short of the concentra-

tion needed for osmotic balance, our guess is that the changes in glycerol content are partly compensated by changes in other solutes (perhaps ions, or trehalose).

The immediate response of \textit{S. cerevisiae} to a salt stress has a more complex pattern than we had previously recognized or suspected. We reported previously [8] that glycerol accumulation begins immediately after transfer of exponential phase \textit{S. cerevisiae} from a conventional broth culture to one containing NaCl (10% w/v) and continues progressively thereafter. This result was obtained over an extended period with the first samples taken at 0, 2.0 and 20.5 h after transfer.

More recent experiments using a synthetic medium and a shorter time scale, however, gave a very different impression (Fig. 1). In this case the initial accumulation of glycerol that normally occurred when the yeast was transferred to a fresh medium was both retarded and greatly diminished in magnitude by salt and the `true' osmoregulatory response did not begin for about 6 h. So when does the `signal' act? Immediately with a delayed response or not for 6 h? As yet we do not know.

5. DISTINCTION BETWEEN OSMOREGULATORY AND COMPATIBLE SOLUTES

In the organisms discussed so far, glycerol has both of these functions. \textit{S. rouxii}, however, also accumulates arabitol, which necessarily contributes to the total osmotic status of the cell but which does not respond appreciably to water stress [5,8]. A somewhat similar situation exists with \textit{D. hansenii} [6]. We have always assumed that the chemical characteristics of arabitol enable it to act as a reserve or supplementary compatible solute if needed and the experiments of Edgley and Brown [8] support this assumption. Some recent experiments of a different type have clearly demonstrated the compatible or protective nature of some non-osmoregulatory solutes and their importance in sustaining viability in a sudden solute stress.

When \textit{S. cerevisiae} is cultivated through a growth cycle in a conventional liquid medium and
plated out at intervals onto malt-extract agar (MA, a conventional high $a_w$ plating medium) and also onto any of several stressing (low $a_w$) plating media, a response of the kind shown in Fig. 2 is obtained. Depending on the details of the experimental conditions, one cell in $10^3$–$10^6$ can form colonies on the stressing agar when transferred from mid-exponential phase. The culture becomes more resistant as it ages and, when fully into stationary phase, there is little or no difference between plate counts on MA and on ‘synthetic honey agar’ (SHA, see Fig. 2). We have called this phenomenon ‘water stress plating hypersensitivity’ and it will be discussed in detail elsewhere (MacKenzie et al., in preparation). *S. rouxii* is not sensitive in this way, nor indeed are some 13 other species we tested. It was encountered only in strains of *S. cerevisiae* and in *Candida krusei*.

Other important characteristics of the phenomenon are: (i) the low plate count on the stressing medium is attributable to death, not dormancy; (ii) the heterogeneity in the population that produces this result is physiological, not genetic; (iii) the ‘plating discrepancy’ (log MA count - log count on stressing agar) has a complex relation with $a_w$ of the stressing medium. For example, in plating media adjusted with glucose, such as SHA, there is no discrepancy below 30% (w/v) glucose and there is approximate proportionality between plating discrepancy and glucose concentration above that; (iv) in continuous culture the plating discrepancy is less at low than at high dilution rates; (v) the culture can readily adapt to effectively complete resistance to the plating stress. For example, growth in 2% (w/v) NaCl will confer resistance to plating on 8–10% (w/v) NaCl or on SHA. (It is of considerable interest to us that, as early as 1940, Doudoroff [12] reported a somewhat similar overall phenomenon in bacteria).

The explanation of these observations seems to be as follows: The resistant species, when growing exponentially in a conventional medium, all accumulate intracellularly one or more polyols (up to 6 C-atoms) to a level above about 0.02 mmol·g$^{-1}$ dry yeast. The sensitive strains do not. The adaptation of *S. cerevisiae* by growing in a low concentration of NaCl is accompanied by the accumulation of glycerol. This is an osmoregulatory response, but the protection so conferred is effective at a glycerol content substantially less than that needed for complete osmotic balance. The resistance acquired by *S. cerevisiae* in stationary phase (and presumably at low dilution rates in continuous culture) is associated with accumulation of the storage carbohydrate, trehalose, which reaches 12–13% of the mass of the dry yeast. This is not an osmoregulatory phenomenon but a biochemical consequence of the changing dynamics of carbohydrate metabolism [e.g., 13–15]. Fig. 3 shows the behavior under relevant conditions of a trehalose-negative mutant of *S. cerevisiae*. Other biochemical evidence for the role of trehalose will be presented elsewhere (MacKenzie et al., in preparation), although we suspect that there is an additional factor(s) that might also contribute to some extent to stationary phase resistance.

To summarize, then, the roles of the various
polyhydroxylic compounds in protecting yeast against the potentially lethal effects of a sudden solute stress are as follows:

(1) protection is conferred by an inherent (i.e., non-osmoregulatory) accumulation of a polyol (we assume that any naturally-occurring polyol will do);

(2) protection is conferred by the adaptive (i.e., osmoregulatory) accumulation of glycerol to a level substantially less than that needed for complete osmotic balance;

(3) protection is conferred by the (non-osmoregulatory) accumulation of the storage polysaccharide, trehalose.

In each of these circumstances the polyhydroxylic compound is a compatible solute. Only in (2) is it also an osmoregulatory solute.

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REFERENCES

SHORT COMMUNICATION

Water Stress Plating Hypersensitivity of Yeasts

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Saccharomyces cerevisiae, when growing exponentially in batch culture, passed through a phase in which, on average, one cell in 10^4 survived plating onto a low water activity (a_w) agar medium. Stationary phase cultures were resistant as were all other species tested, with the exception of Candida krusei. In continuous culture, S. cerevisiae was more resistant at low than at high dilution rates. Plating at low a_w was lethal to those cells that were not protected by an adequate content of compatible solute. In naturally resistant yeasts and in S. cerevisiae that had been exposed to an adaptation process, the compatible solute was one or more types of polyhydric alcohol. Resistance in stationary phase was attributable to a different cause.

INTRODUCTION

Edgley & Brown (1983) reported major differences in the process of adaptation from a conventional broth medium to one containing NaCl (10%, w/v) between Saccharomyces cerevisiae and the xerotolerant species Saccharomyces rouxii. The differences included a dramatic drop in apparent viability by the former yeast but not by the latter. The apparent loss in viability by S. cerevisiae complicated interpretation of the adaptation sequence, in particular whether adaptation was achieved essentially by the whole population or a small residue that survived during the transition. The present paper describes a peculiarity of population dynamics that is relevant to that question. We have called this phenomenon 'water stress plating hypersensitivity'.

METHODS

Organisms. S. rouxii and S. cerevisiae were maintained as previously described (Edgley & Brown, 1983). Candida albicans, C. glabrata, C. krusei and C. parapsilosis were obtained from W. Crozier (Wollongong Hospital, Australia); S. bailii and Pichia membranefaciens from J. Pitt (CSIRO Food Research Labs, North Ryde, Australia); P. miso from H. Onishi (Dept. of Agricultural Chemistry, Kagoshima University, Japan) and Debaryomyces hansenii from L. Adler (Dept. of Marine Microbiology, University of Gothenburg, Sweden). Saccharomyces carlsbergensis and S. cerevisiae var. ellipsoideus were from our own culture collection. For experimental purposes, liquid batch cultures were grown as previously described (Edgley & Brown, 1983) in basal yeast medium (BYM), a conventional high a_w broth, or with modifications as indicated in Results.

Counting. Samples for total counts and plating were first treated in a glass/Teflon homogenizer (diameter 16 mm) to disaggregate clumps. A sample of 3 ml was subjected to 75 double strokes of the Teflon plunger (about 2 min). Total counts were made conventionally in a counting chamber. A colony-forming unit (c.f.u.) is defined as any cell or aggregate of cells which, if viable, would produce a single colony.

Plating media were used according to Edgley & Brown (1983) except that pour plates were used instead of drop plates. This change was introduced because yeast suspensions adapted to a lower a_w during the drying of a drop on

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Abbreviations: BYM, basal yeast medium; MA, malt agar; SHA, synthetic honey agar.
Short communication

a 'synthetic honey agar' plate (SHA, containing 48%, w/v, glucose, a₀ 0.924). Plates were seeded with 0.1 ml suspension and poured with 10 ml agar. They were incubated at 30 °C inside plastic bags to restrict evaporation. There were no qualitative differences of consequence between results obtained with a plating medium containing NaCl (8 or 10%, w/v) and one with 48% glucose (SHA; see Fig. 1). SHA was used routinely since colonies could be counted after 6 d compared with about three weeks for NaCl agar.

Polyol chromatography. Freeze-dried yeast was extracted with ethanol (Edgley & Brown, 1983). Polyols were analysed by thin-layer chromatography on silica gel with propan-1-ol/concentrated NH₄OH/water (6:2:1, by vol.) and with butan-1-ol/acetic acid/water (6:1:2, by vol.) as developing solvents. They were detected with a periodate-Schiff base reagent (Baddiley et al., 1956) and with alkaline silver nitrate. Glycerol was estimated as described by Edgley & Brown (1983).

RESULTS AND DISCUSSION

General characteristics

During the course of a growth cycle in BYM, S. cerevisiae gave plate counts on malt agar (MA) and various low a₀ stressing agars as shown in Fig. 1. The essential characteristic of the phenomenon was a huge drop in colony-forming ability on a stressing agar during the period of exponential growth. The population developed resistance as it entered stationary phase. Since stationary phase inocula were always used, the culture was also resistant at the beginning of the growth cycle.

At its maximum, the plating discrepancy (log MA count — log stressing agar count) was, for Fig. 1, about 2.5–4 but, under some conditions, discrepancies up to 6 were obtained. In other words, 1 c.f.u. in 10⁶ is viable on the stressing agar under such extreme conditions. Plate counts on NaCl agar were not increased by inclusion in the agar of glycine betaine (0.25–0.5 mM) or glycerol (0.5–1.0 mM), agents capable of relieving solute stress under some conditions (cf. Le Rudulier et al., 1984). Since early results indicated a close correlation between plating discrepancy and budding, we considered a mechanism in which a specific step in the cell division cycle was critically sensitive to the solute stress. The correlation was subsequently found to be superficial and strongly dependent on experimental conditions. When S. cerevisiae was grown in BYM in continuous culture, the plating discrepancy was about 1 for dilution rates in the range 0.02–0.05 h⁻¹ and about 3 in the range 0.1–0.13 h⁻¹ with a transition between the ranges.

Distribution

Fourteen species and strains (see Methods) were screened. Plating hypersensitivity was found only in strains of S. cerevisiae, including var. ellipsoideus, and in Candida krusei. None of the sensitive yeasts, when harvested from mid-exponential phase in BYM, had accumulated polyols that were detected on chromatograms [≥ 0.02 mmol (g dry yeast)⁻¹, for glycerol]. Every resistant yeast accumulated one or more of the polyols glycerol, erythritol, arabitol or mannitol in readily detectable quantities. Accumulation of arabitol by D. hansenii and S. rouxii had already been established (Adler & Gustafsson, 1980; Brown, 1978). Both haploid and diploid strains were included in the comparison; ploidy did not correlate with sensitivity.

Physiological characteristics

The heterogeneity of the population responsible for the results in Fig. 1 was physiological, not genetic, inasmuch as resistant colonies isolated from mid-exponential phase SHA plates gave rise to cultures with identical characteristics. Moreover, failure to grow on SHA was attributable to death, not dormancy. This was demonstrated by two versions of an experiment in which either excised areas or whole plates of SHA were incubated in quarter-strength Ringer's solution at various time intervals after the plate was poured. Comparisons with appropriate controls showed clearly that, by this criterion, those cells that did not form colonies had died and were not merely suppressed.

The relation between plating discrepancy and a₀ (or, in Fig. 2, glucose concentration) was complex. There was no discrepancy below 30% (w/v) glucose but there was approximate proportionality between the two variables at higher glucose concentrations (Fig. 2).
Growth of *S. cerevisiae* in BYM containing NaCl eliminated plating hypersensitivity but there was a pronounced effect of NaCl concentration. There was no plating discrepancy with cultures grown in media containing 2% (w/v) or more NaCl; below 2%, plating discrepancy increased sharply with decreasing NaCl concentration. Furthermore, washing (three times by centrifugation in 0.01 M-potassium phosphate, pH 6.5) substantially reversed the adaptation. Adaptation was accompanied by accumulation of glycerol (from <0.05 mmol g⁻¹ for yeast grown in BYM to 4 mmol g⁻¹ when grown in 4% NaCl). Washing removed 80% of the glycerol from yeast grown in 2% NaCl. The yeast also adapted within about 1 h when incubated in a simple NaCl solution (i.e. no nutrient) at concentrations of 1–4% (w/v). Here, too, adaptation was complete in 2% NaCl. Adaptation was thus a bulk phenomenon, not selection of an inherently resistant variant.

Moreover the amount of intracellular glycerol that accumulated in response to growth in 2% NaCl (about 0.99 a₅₀) was substantially less than that needed for complete osmotic adjustment to SHA (0.924 a₅₀). This suggests that the protection conferred by the accumulated glycerol was achieved primarily through its role as a compatible solute rather than by the osmoregulatory process of promoting complete turgor adjustment.

The inherent resistance of the insensitive strains and the acquired resistance of *S. cerevisiae* on adaptation are each attributable to polyol accumulation and the function of such compounds as compatible solutes. The resistance developed by *S. cerevisiae* in stationary phase is largely, but probably not wholly, attributable to the accumulation of the storage polysaccharide trehalose (Brown et al., 1986); this is not an osmoregulatory process.

Water stress plating hypersensitivity is strikingly similar in several respects to the adaptation of *Escherichia coli* to NaCl as described by Doudoroff (1940).

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Water Stress Plating Hypersensitivity of Yeasts: Protective Role of Trehalose in Saccharomyces cerevisiae

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Water stress plating hypersensitivity was studied in two strains of Saccharomyces cerevisiae, one of them being a mutant incapable of accumulating trehalose to significant levels. The wild-type strain was grown in a defined medium with glucose, maltose or ethanol as carbon/energy source. In each case plating hypersensitivity was demonstrated and resistance to the stress developed in the second half of the exponential growth phase. Development of resistance was accompanied by accumulation of trehalose and was apparently unrelated to glycerol content which, under these conditions, was always low. A qualitatively similar trend was observed in the mutant grown on glucose but trehalose levels remained low and recovery of stress resistance was only slight. Dinitrophenol induced trehalose breakdown in resting yeast and simultaneously induced the onset of plating hypersensitivity. A negative correlation was demonstrated between trehalose content and 'plating discrepancy' (log colony count on 'normal' agar - log colony count on stressing agar) for both strains under all experimental conditions. The correlation held for trehalose contents up to about 50 mg (g dry yeast)^{-1}, above which the yeasts were apparently fully resistant. Trehalose was evidently a more effective compatible solute, per mole, than glycerol.

INTRODUCTION

The phenomenon that we have called 'water stress plating hypersensitivity' (Brown et al., 1986; Mackenzie et al., 1986) is demonstrated by viable counts during growth of a sensitive yeast such as Saccharomyces cerevisiae in a conventional high water activity (a_w) liquid growth medium. When plate counts are made on both a high a_w nutrient agar medium and one of low a_w such as 'synthetic honey agar' (containing 48%, w/v, glucose), there is a discrepancy of some 3-4 logarithms between the counts on the two media in mid-exponential phase. In stationary phase the discrepancy diminishes, ultimately and usually to insignificance. The magnitude of the plating discrepancy is a function of the solute concentration (or a_w) of the low a_w plating medium. At solute concentrations above a critical threshold value, the discrepancy is roughly proportional to the solute concentration; below the threshold there is no discrepancy (Mackenzie et al., 1986).

Xerotolerant yeasts do not have the characteristic nor do some others that would not normally be considered xerotolerant. Plating hypersensitivity was found in only three of fourteen species and strains of yeasts screened. All resistant strains accumulated significant quantities of at least one polyhydric alcohol while growing exponentially in a conventional high a_w broth. Sensitive strains did not and we concluded from this and other evidence that the compatible solute properties of the polyols were sufficient to protect against the potentially lethal effects of the sudden dehydration incurred by plating into a stressing agar medium (Mackenzie et al., 1986).

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Abbreviations: BYM, basal yeast medium; MA, malt-extract agar; SHA, synthetic honey agar.

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Protection by polyols, however, did not explain the recovery of resistance by sensitive strains on entering stationary phase of a growth cycle. Many yeasts and fungi, when grown in laboratory batch culture, accumulate trehalose as the culture ages; the accumulation is a consequence of a change in the dynamics and regulation of carbohydrate metabolism (Küenzi & Fiechter, 1972; Fiechter et al., 1981; Thevelein, 1984a). Our strain of S. cerevisiae is no exception and we have already presented some evidence that trehalose confers resistance against a sudden severe solute stress (Brown et al., 1986). The present paper adds to that evidence.

METHODS

Organisms. Saccharomyces cerevisiae (strain Y41, ATCC 38531) and S. cerevisiae (strain 212-244-1A) (Yeast Genetic Stock Center, University of California, Berkeley, USA) were maintained and cultured according to Edgley & Brown (1983): they were counted according to Mackenzie et al. (1986). Two liquid culture media were used, namely basal yeast medium (BYM, a conventional broth, 0.997 aw, ψw = −0.42 MPa at 30°C), and Wickerham’s synthetic medium (Barnett et al., 1983), of approximately the same aw. This medium, without the carbon source. Samples (200 ml) were filtered aseptically through a membrane (0.8 μm pore size) and then before use. The carbon source was mostly used at a final concentration of 0.5% (w/v). The plating media used were malt extract agar (MA, conventional complex high aw medium) and ‘synthetic honey agar’ (SHA, 48%, w/v, glucose, aw 0.924, ψw = −11.1 MPa at 30°C; Anand & Brown, 1968). In experiments in which strain Y41 was exposed to dinitrophenol (DNP), the yeast was first grown for 72 h in Wickerham’s medium with glucose as the carbon source. Samples (200 ml) were filtered aseptically through a membrane (0.8 μm pore size) and then transferred, with the filter, to fresh synthetic medium (200 ml) without a carbon source, with and without DNP (2 mM).

Analytical methods. Samples (10 ml) of culture suspension were withdrawn and filtered rapidly under vacuum through a cellulose acetate membrane filter (0.8 μm pore size). If trehalose were to be estimated in yeast sampled from media containing either glucose or maltose, the yeast was washed twice on the filter with potassium phosphate buffer (pH 5.9), isotonic with the growth medium. Otherwise the yeast was not washed. The yeast was then frozen in liquid nitrogen, freeze-dried and stored at −80°C pending extraction. Filtrates were also frozen in liquid nitrogen and stored at −80°C for periods of up to 4 weeks until analyzed. Freeze-dried yeast was extracted with ethanol according to Edgley & Brown (1983). For comparative purposes trehalose was also estimated in trichloroacetic acid (0.5 M) extracts (Trevelyan & Harrison, 1952) but there was no significant difference between the two methods of extraction. Approximately 1 ml ethanol or TCA solution was used per 4 mg dry yeast. Ethanolic extracts were dried under vacuum, redissolved in water (1.0 ml) and stored at −80°C. Trichloroacetic acid extracts were treated according to Trevelyan & Harrison (1952), adjusted to 1.0 ml and stored at −80°C.

Ethanol was estimated (in filtrates only) according to Beutler (1984). Glucose and glycerol were estimated as described previously (Edgley & Brown, 1983). Trehalose was estimated by reaction with anthrone (Stewart, 1975) in 0.2 ml volumes of cell extract.

RESULTS AND DISCUSSION

When S. cerevisiae was grown at 15°C in BYM it had essentially the same characteristics of plating hypersensitivity (not illustrated) as described previously (Mackenzie et al., 1986) for growth at 30°C. This contrasts with the correlation previously observed (Mackenzie et al., 1986) between nutrient-limited growth rate in continuous culture and plating discrepancy (log MA plate count − log low aw plate count). Evidently, survival of exponential phase yeast after a sudden solute stress is affected not simply by growth rate itself but rather by the factors that limit growth rate. Nutrient limitation in continuous culture, like the cessation of exponential growth in batch culture, is effective whereas a general suppression of all reaction rates is not.

Growth at 30°C in synthetic medium with glucose, maltose or ethanol as carbon source all gave growth curves with typical water stress plating hypersensitivity. Fig. 1 shows results of a series of assays on S. cerevisiae (Y41) grown with glucose as the carbon source. Exhaustion of glucose, the start of measurable consumption of ethanol, the maximum in the proportion of budded yeast cells and the minimum in the SHA plate count all happened within 4–6 h of inoculation. Both trehalose and glycerol content fell initially; trehalose reached an apparent minimum about 8 h after inoculation. Essentially similar results were obtained when either ethanol or maltose was the carbon source even though maltose, in contrast to glucose, has been reported to sustain trehalose accumulation during exponential growth (Panek et al., 1978).

When stationary phase yeast is transferred to fresh medium, the accumulated trehalose is
Trehalose as a compatible solute in yeast

Fig. 1. Partial growth cycle of \textit{S. cerevisiae} strain Y41 on synthetic medium with glucose as carbon/energy source. (a) Proportion of c.f.u. with buds. (b) \(\nabla\), Total count; \(\blacktriangle\), MA viable count; \(\blacklozenge\), SHA viable count. (c) Glucose (□) and ethanol (◇) concentration in the medium. (A corresponding plot for glycerol concentration had essentially the same shape as that for ethanol but with a maximal glycerol concentration of about 2.5 mM.) (d) Intracellular glycerol (♦) and trehalose (▵). Points represent the means of two separate experiments; in (b) the means of the logarithms are used.

Fig. 2. Effect of DNP (2.0 mM) on the viability, water stress plating hypersensitivity and trehalose content of resting \textit{S. cerevisiae} strain Y41 suspended in synthetic medium without a carbon/energy source. (a), Viable counts: \(\blacktriangle\), MA colony counts; \(\blacklozenge\), SHA colony counts. \(\blacktriangle\), \(\blacklozenge\), Suspensions without DNP; \(\blacktriangle\), \(\blacklozenge\), suspensions with DNP. (b) Trehalose content: \(\blacklozenge\), suspension without DNP; \(\blacklozenge\), suspension with DNP.

Trehalose is rapidly hydrolysed by trehalase, which is activated by glucose metabolism. The mechanism usually ascribed to the activation process involves phosphorylation of the enzyme protein by cyclic AMP (e.g. Thevelein & Beullens, 1985).

Membrane depolarizing agents such as DNP stimulate trehalose breakdown in yeast; this has also been attributed to activation of trehalase by phosphorylation (Thevelein, 1984b). On the other hand, Valle \textit{et al.} (1986) have presented evidence that activation of trehalase can be attributed to acidification of the cytoplasm rather than to membrane depolarization. Whatever the mechanism, however, there is ample empirical evidence that DNP stimulates the breakdown of trehalose in resting yeast. Fig. 2 shows that exposure of strain Y41 to DNP did not
Fig. 3. Partial growth cycle of *S. cerevisiae* strain 212-244-1A (*glc1*, trehalose negative) on synthetic medium with glucose as carbon/energy source. (a) Proportion of c.f.u. with buds. (b) Total count; MA viable count; SHA viable count. (c) Intracellular glycerol (♦) and trehalose (▲). Points represent the means and SD of three separate experiments; in (b) the mean of the logarithm is used. Where error bars are not shown the SD lay within the symbols. Note the difference in the trehalose scale between this Figure and Fig. 1.

Fig. 4. Plating discrepancy vs trehalose content for strain Y41 grown in synthetic medium on glucose (●), maltose (▲) and ethanol (♦) and strain 212-244-1A grown on glucose (□). The three boxed-in points were excluded from the calculation of the regression as there were the two open circles (arrowed) which denote values for strain Y41 at two different dilution rates in complex medium in continuous culture. The regression line has a slope of -0.084 and a correlation coefficient of -0.94. Other interpretations are possible (see text).

affect viability on MA but did promote a decrease in both viability on SHA and trehalose content, although there were differences in the patterns of change.

Additional evidence for a protective role of trehalose was provided by strain 212-244-1A, which carries the *glc1* mutation, a regulatory pleiotropic mutation that diminishes the accumulation of both trehalose and glycogen. Trehalose accumulation is prevented because trehalase always remains in its phosphorylated active form to a significant extent (Ortiz *et al.*, 1983; Tenan *et al.*, 1985). We reported previously preliminary results indicating that strain 212-244-1A grown in complex medium does not recover from its hypersensitive state on entering stationary phase (Brown *et al.*, 1986). Fig. 3 shows results of a similar but more comprehensive experiment in which the *glc1* mutant strain was grown throughout in synthetic medium.

The total count rose to a slightly higher level than did that of strain Y41 in similar circumstances (Fig. 1) but, although the SHA count oscillated throughout the growth cycle, it did not recover in stationary phase; the plating discrepancy remained between 2.9 and 3.9 throughout the growth cycle. The concentrations of glucose, ethanol and glycero in the medium were similar to those produced by Y41 (Fig. 1) and are not illustrated. The changes in intracellular glycerol content, however, were different from those encountered in strain Y41.
There was a peak in glycerol content after 15-20 h when the organism had entered stationary phase. This corresponded closely with a peak in the SHA plate count. There was no such peak with Y41 although there was some evidence of a small shoulder after about 10 h. Moreover, although the maximal glycerol content was higher in Y41, glycerol content remained higher in the glcl strain late in the growth cycle.

The pattern of trehalose content throughout the growth cycle was similar to that of Y41 inasmuch as there was a minimum after about 5 h (Fig. 3) but the subsequent course of accumulation was different in the two strains. The highest value encountered in 212-244-1A was slightly over 0.042 mmol (g dry yeast)$^{-1}$ (1.5%) compared with 0.21 mmol g$^{-1}$ (7.5%) for Y41.

The quantitative relation between trehalose content and plating discrepancy is shown in Fig. 4 for both strain 212-244-1A grown on glucose and strain Y41 grown separately on glucose, maltose, and ethanol. An alternative interpretation of these results is that the relation between plating discrepancy and trehalose content is curved. A linear regression with a correlation coefficient of $-0.91$ can be fitted to a plot of plating discrepancy vs log trehalose content (with the boxed points included). (The inclusion of the boxed points in the arithmetic relation numerically lowers the correlation coefficient to $-0.90$.) From theoretical considerations, however, we consider that Fig. 4 represents the most likely quantitative interpretation of the results. It implies a negative linear relation between plating discrepancy and mean trehalose content up to a value of about 50 mg (g dry yeast)$^{-1}$. At higher levels, the entire population is apparently resistant.

For mean trehalose contents of less than 50 mg g$^{-1}$, the mathematical significance of Fig. 4 is as follows:

$$\Delta PD = -0.084 \Delta T$$

where $PD$ denotes plating discrepancy and $T$ the trehalose content of the yeast. By definition, $PD = \log M - \log S$, where $M$ is the MA plate count and $S$ the SHA plate count. Since $M$ is independent of trehalose content, $\Delta \log M = 0$ and therefore

$$-\Delta \log S = -0.084 \Delta T$$

or

$$\Delta \log S = 0.084 \Delta T$$

This equation will not be valid for a stress of a magnitude different from that used in these experiments and might not be valid for different culture conditions. Nevertheless, the values obtained from a complex medium in continuous culture lay close to the regression line (Fig. 4).

In practical terms, equation (2) means that a change of 1 mg g$^{-1}$ in the mean trehalose content of a yeast population will be accompanied by a change in the number of survivors on SHA of 0.084 logarithms (1.2 fold). The trehalose analyses, however, represent mean values for the bulk population whereas the plate counts, which are digital measurements, divide the population into two groups, viable and non-viable. If trehalose is, in fact, the principal protective agent then it would be expected to be distributed non-uniformly in the population; for our immediate purpose, its distribution can be expressed as values either above or below a critical threshold level. Fig. 4 indicates that the entire population was protected at a minimum mean value of about 50 mg trehalose (g dry yeast)$^{-1}$. At levels higher than this, the population was demonstrably homogeneous with respect to stress resistance and therefore we can assume that, under such conditions, trehalose is more uniformly distributed than it is in the linearly responsive part of Fig. 4. Hence 50 mg g$^{-1}$ (or 0.15 mmol g$^{-1}$) should approximate the critical threshold value for a single yeast cell. Under these conditions trehalose is thus a more effective compatible solute than glycerol which confers complete protection at about 2 mmol (g dry yeast)$^{-1}$ (Mackenzie et al., 1986). Micro-organisms generally are more resistant to chemical and physical stresses when resting than when multiplying rapidly. It is unlikely that trehalose is the only source of resistance to solute stress in stationary phase yeast, even though there is no evidence from our results for a major direct contribution by any other mechanism.

Trehalose has also been reported to accumulate in *S. cerevisiae* in response to heat under which conditions it protects against both heat shock and desiccation (Hottiger et al., 1987). All
compatible solutes might not have the same mode of action, however. For example, trehalose is especially effective in stabilizing lipoprotein membranes and lipid monolayers (Crowe et al., 1984a, b), whereas glycerol is notable for protecting soluble enzymes (see Brown, 1978).

Trehalose has been reported to be an osmoregulatory solute in some prokaryotes (e.g. MacKay et al., 1984; Strom et al., 1986; Larsen et al., 1987); it necessarily contributes to the overall osmotic status of yeast but it is far from clear that it has an osmoregulatory function. Edgley (1980) reported an increase in trehalose content of S. cerevisiae grown at an elevated salt concentration, but the dependence of trehalose accumulation on a low energy flux is sufficient to deny it a significant role as an osmoregulator in a strict sense.

REFERENCES


