Influence of Solute and Hydrogen Ion Concentration on the Water Relations of some Xerophilic Fungi

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SUMMARY

Germination and growth of six xerophilic fungi, *Aspergillus flavus*, *Aspergillus ochraceus*, *Eurotium chevalieri*, *Chrysosporium fastidium*, *Wallemia sebi* and *Xeromyces bisporus* were examined on media of a wide range of water activities (a_w). The influence of three solutes, NaCl, glycerol and a glucose/fructose mixture, was studied at pH 4.0 and pH 6.5 using a plate-slide technique. Germination times and growth rates were affected by solute type, but the influence of pH was less marked. Except for *Wallemia sebi*, the fungi grew most strongly on glucose/fructose and were partially or completely inhibited by NaCl. The results showed that a universal isolation medium for xerophilic fungi could be based on glycerol or glucose/fructose but not on NaCl as a_w -limiting solute.

INTRODUCTION

The growth of fungi under conditions where water availability is a limiting factor is controlled primarily by the water activity (a_w) (Scott, 1957). Although other factors such as pH of the medium and the solutes present are also known to be influential, available data are fragmentary (Pitt, 1975). The present series of experiments was designed to compare the effect of solute and pH on the germination and growth of several diverse xerophilic fungi.

Pitt (1975) pointed out the need for a medium universally suitable for the isolation of xerophilic fungi from foods and other commodities. This study was designed in part to aid development of such a medium.

A novel procedure was used for studying the germination and growth of the xerophilic fungi. Richter & Amsterdam (1972) reported that polystyrene plate-slides (Petrislides, manufactured by Millipore Corporation) were well suited to the observation of growing fungal colonies. Using media of known a_w in the Petrislides, their method was adapted to the study of germination and growth rates of xerophiles.

METHODS

Fungi. Six xerophilic fungal isolates were studied: *Aspergillus flavus* Link ex Fr. FRR1449, from contaminated milk powder, 1973; *Aspergillus ochraceus* Wilhelm FRR1588, isolated by M. D. Connole from cattle feed, 1974; *Chrysosporium fastidium* Pitt, type isolate, FRR77, from prunes, 1964; *Eurotium chevalieri* Mangin FRR1311, from prunes, 1962; *Wallemia sebi* (Fries) v. Arx FRR1473, from bread; and *Xeromyces bisporus* Fraser [= *Monascus bisporus* (Fraser) v. Arx] FRR1522, from spoiled licorice, 1973. FRR denotes the culture collection of the CSIRO Division of Food Research, North Ryde, N.S.W., Australia.

Media. The basal medium was 0.67% Yeast Nitrogen Base (Difco) plus 2% glucose and 2% agar (all w/v). For one series of experiments this was buffered to about pH 4.0 with

0.59% succinic acid and 0.125% NaOH; for the second to about pH 6.5 with 0.4% K_2HPO_4 (all w/v). The two pH values were chosen as representing values likely to be encountered in foods of interest and suitable for use in making isolation media.

Media of various water activities were prepared by adding NaCl or glycerol or a mixture of equal weights of glucose and fructose. Analytical grade chemicals were used throughout. Twelve media were prepared which contained NaCl as controlling solute, the appropriate concentrations being calculated from the data of Robinson & Stokes (1955). Water activities ranged from 0.997 to 0.753. From the formulae of Norrish (1966), 16 glucose/fructose and 14 glycerol media were prepared with a_w values ranging from 0.997 to 0.600 and 0.997 to 0.667, respectively. As the addition of high concentrations of solutes produced appreciable pH changes, all media were finally adjusted to pH 4.0 ± 0.1 or 6.5 ± 0.1 , as required, by adding small amounts of 10% NaOH or 10% HCl (both w/v) just before use. Media of pH 6.5 and a_w greater than 0.92 were sterilized by autoclaving and the remainder by steam at 100 °C.

Actual a_w values for media of 0.94 a_w and below were checked with a Sina-Scope instrument (Sina, Zurich, Switzerland). Corrected values, which rarely varied by more than 0.01 a_w from those calculated, have been used below.

Cultivation. Petrislides (Millipore) containing 2 ml medium were used for growth and examination of the fungi. Each inoculum was a needlepoint of mature spores, placed at the centre of the chamber: if necessary inoculation was carried out by holding the Petrislide inverted to minimize scattering of the spore inoculum.

To minimize water transfer to or from the media, inoculated Petrislides were stacked in polyethylene food storage boxes containing dishes of appropriate saturated salt solutions (Robinson & Stokes, 1955). Incubation was at 25 °C for a maximum period of 100 days.

Examination. To observe germination, Petrislides were examined regularly (initially twice daily, ultimately weekly) by transmitted light microscopy at 100 $\times$ magnification. The criterion for germination was the observation of a significant number of germ tubes of length equal to the diameter of the spores examined. Colony diameters were measured at intervals (daily to weekly), initially by an eyepiece micrometer and subsequently by stage verniers. From these data radial growth rates, expressed as $\mu m h^{-1}$, were calculated for each time increment. For the period over which growth was approximately linear (Brancato & Golding, 1953), a mean radial growth rate was calculated for each fungus and set of conditions. Early exponential growth (Trinci, 1971) and late suboptimal growth were not included.

RESULTS

Germination time

Representative data for germination times are shown in Fig. 1, in which log reciprocal germination time is plotted against a_w , a technique first used by Snow (1949). Data for four fungi are shown: although lower a_w limits differed somewhat, *Aspergillus ochraceus* behaved similarly to *A. flavus*, and *Wallemia sebi* to *Eurotium chevalieri*.

Germination of *A. flavus* and *A. ochraceus* was little affected by pH or solute. For the other fungi, glucose and glycerol were equally favourable substrates, except that *Xeromyces bisporus* was somewhat intolerant of glycerol at low a_w . However, NaCl caused marked inhibition of *E. chevalieri* at a_w below 0.90 and of *Chrysosporium fastidium* at a_w below 0.98. At pH 6.5, *X. bisporus* failed to germinate at any a_w in the presence of NaCl.

The relationship between germination time and a_w varied widely. Germination times for *A. flavus* and *A. ochraceus* were shortest at the highest a_w used (0.997), while *X. bisporus* and

Fig. 1. Effect of a_w and solute on the time taken (t) for germination of spores of four xerophilic fungi: (a) *Aspergillus flavus*; (b) *Eurotium chevalieri*; (c) *Xeromyces bisporus*; (d) *Chrysosporium fastidium*. (●) NaCl, pH 4.0; (○) NaCl, pH 6.5; (■) glucose/fructose, pH 4.0; (□) glucose/fructose, pH 6.5; (▲) glycerol, pH 4.0; (△) glycerol, pH 6.5.

Table 1. Minimum a_w for germination of *Aspergillus ochraceus* and *Wallemia sebi*

Solute	pH	<i>A. ochraceus</i>		<i>W. sebi</i>	
		Minimum a_w	Germination time (days)	Minimum a_w	Germination time (days)
NaCl	4.0	0.839	6.0	0.805	5.8
	6.5	0.805	69	0.751	12
Glucose/fructose	4.0	0.792	12	0.691	38
	6.5	0.787	21	0.711	42
Glycerol	4.0	0.806	8.3	0.806	4.8
	6.5	0.800*	7.5	0.700*	25

* Nominal a_w of medium in the absence of Sina-Scope data.

Fig. 2. Effect of a_w and solute on the radial growth rate of four xerophilic fungi: (a) *Aspergillus flavus*; (b) *Eurotium chevalieri*; (c) *Xeromyces bisporus*; (d) *Chrysosporium fastidium*. (●) NaCl, pH 4.0; (○) NaCl, pH 6.5; (■) glucose/fructose, pH 4.0; (□) glucose/fructose, pH 6.5; (▲) glycerol, pH 4.0; (△) glycerol, pH 6.5.

C. fastidium germinated most rapidly at much lower a_w values. *Xeromyces bisporus* failed to germinate above 0.96 a_w , and germination times remained almost constant over the entire range of 0.94 to 0.80 a_w .

Minimum a_w for germination

At high a_w inhibitory effects of pH or solute usually resulted in extended germination times, but at lower a_w complete inhibition often occurred. The minimum a_w for germination therefore varied widely, as illustrated in Table 1 and Fig. 1. In media containing NaCl, *C. fastidium* failed to germinate below 0.92 a_w , but did germinate at 0.7 a_w or below in the other systems. The contrast between NaCl and the other solutes was even more marked for *X. bisporus*. The other fungi were much less affected, although *W. sebi* was relatively intolerant of pH 4 in both glycerol and NaCl.

Colony growth rates

Mean radial growth rates for the same four fungi as in Fig. 1 are shown in Fig. 2: curves for *A. ochraceus* were qualitatively similar to those for *A. flavus*, while growth of *W. sebi* was very slow under all conditions.

The effects of solute and pH on growth were generally similar to those on germination. *Aspergillus flavus* showed the least variation in response. Even so, at the optimum a_w for growth (0.98), the radial growth rate on glycerol at pH 6.5 was less than 80% of that on NaCl at pH 6.5 or glucose/fructose at pH 4, while at 0.90 a_w the growth rate on NaCl was little more than 20% of that in glucose/fructose at either pH.

At the other extreme, *X. bisporus* and *C. fastidium* were markedly intolerant of NaCl. At 0.90 to 0.92 a_w and pH 4.0, the growth rate was only 10% of that in glucose/fructose. As noted above, *X. bisporus* failed to germinate in NaCl at pH 6.5; *C. fastidium* was able to germinate under these conditions, but failed to grow beyond malformed germ tubes.

Unlike the other fungi, *W. sebi* grew best at pH 6.5, and grew more rapidly in NaCl than in the other solutes at this pH.

Minimum a_w for growth

Even under unfavourable conditions, germination was usually followed by growth, although when germination required several weeks, growth was sometimes slow and non-linear during the experiment.

DISCUSSION

The plate-slide technique proved to be satisfactory for water relations studies. As the Petrislides acted as a barrier between the controlling solution and the medium, undesirable changes in a_w due to water generated by growth might occur. However, measurements made with the Sina-Scope at the conclusion of the experiment indicated that such changes were minor. Errors due to water loss during examination under the microscope, an obvious problem with the technique of Pitt & Christian (1968), were effectively reduced by the plate-slide technique. Moreover, ease and clarity of microscopic inspection were improved.

Based on one series of experiments with a single fungus, *Eurotium amstelodami* Mangin, Scott (1957) postulated that the optimum a_w for growth of xerophilic fungi was independent of the predominant solute in the medium. His hypothesis appears to have been generally accepted but with little further experimental evidence to support it. In the main our data support his view. With the exception of growth in NaCl at pH 4.0, a_w optima appeared to vary no more than 0.02 under the conditions examined.

Scott (1957) also noted that the absolute rates of growth over a range of a_w depend on the nature of the solute. The data reported here amplify his statement. The less-specialized fungi, the *Aspergillus* species, were little affected by solute type at either pH over the entire range of a_w at which germination and growth occurred. The more highly adapted fungi, *Xeromyces bisporus* and *Chrysosporium fastidium*, were markedly intolerant of solutes other than glucose/fructose.

Wallemia sebi was reported by Frank & Hess (1941) to have an obligate requirement for NaCl, i.e. to be a halophile. However, Vaisey (1954) showed it to be equally capable of growth at low a_w in other solutes including glucose. Data in Table 1 and other results (not shown) indicate *W. sebi* grows best at neutral pH but has no requirement for NaCl as a solute. Because neither *W. sebi* nor any other fungus has been shown to require NaCl for growth at low a_w , the term halophile is properly restricted to bacteria.

Schmiedeknecht (1960) suggested that xerophiles could be classified by their optimum a_w for growth. Certainly, it is to be expected that the lower the optimum a_w , the more xerophilic a fungus would be. On this basis, the fungi examined here can be placed in order: *Aspergillus flavus* (optimum a_w 0.98), *A. ochraceus* (0.98 to 0.95), *Eurotium chevalieri* (0.96 to 0.94), *Wallemia sebi* (0.94), *Chrysosporium fastidium* (0.92) and *Xeromyces bisporus* (0.86). There is a strong correlation (using $1 - a_w$ as variates, $r^2 = 0.826$) between these optima and the minimum a_w for germination in the most favourable solute.

The findings reported above have important implications for the development of isolation media for xerophiles. The use of media containing high concentrations of NaCl, first suggested by Christensen (1946) and widely used since, is clearly unsatisfactory. Although the other fungi grew well, *C. fastidium* and *X. bisporus* were intolerant of NaCl. The glucose/fructose mixture provided favourable growth conditions for all of the fungi studied. However, the concentrations of the sugars required to produce 0.90 or 0.85 a_w make this medium difficult to handle, because of high viscosity, and relatively expensive. Glycerol, an ideal solute in terms of ease of use and cheapness, is a possible alternative. At 0.90 a_w all of the fungi examined grew reasonably well in this medium, at 30 % or more of the maximum rates achieved. At 0.85 a_w , *W. sebi* grew in the presence of glycerol only at pH 6.5, so a universal medium based on glycerol would need to be of near neutral pH.

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