Heat Resistance of Xerophilic Fungi Based on Microscopical Assessment of Spore Survival

J. I. Pitt and J. H. B. Christian

C. S. I. R. O., Division of Food Preservation, Ryde, New South Wales, 2112, Australia

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An improved viable counting technique was developed to facilitate study of the heat resistance of fungal spores. Spores were heated and subsequently incubated in the same medium. After germination, hyphae and germ tubes were stained with lactofuchsin, and the germinated spores were counted with the aid of a microscope. A number of xerophilic strains were examined, mostly isolated from spoiled high-moisture prunes. Of these, ascospores of *Aspergillus chevalieri*, *A. mangini*, and *Xeromyces bisporus* were the most heat-resistant. A decimal reduction curve obtained for *A. chevalieri* was specified by a z value of 23 F and an F180 of 2.2 min.

To establish a thermal process for the commercial sterilization of high-moisture prunes packed in plastic pouches, information was required on the heat resistance of a group of xerophilic fungi whose isolation and water relations were described previously (11). There are no published data on the heat resistance of these fungi, with the exception of *Xeromyces bisporus* Fraser (3).

The difficulty of obtaining accurate viable counts of fungal spores has led to continued use of thermal death time (TDT) measurements in which only the heating time necessary to destroy all viable cells is measured. This method is much less precise than that commonly used to study bacterial heat resistance, in which the decrease in viability with time is determined quantitatively at each temperature.

Our initial attempts to perform viable counts on fungal spore suspensions were of limited accuracy, as mycelia from rapidly germinating spores obscured those more slowly germinating. The maximum number of mycelia that could be counted on a standard 10-cm petri dish was less than 100, and dilution experiments indicated inaccuracies at this level of up to 20%. For these reasons, a microscopical counting method with greatly increased accuracy was developed.

**MATERIALS AND METHODS**

**Cultures.** The fungi studied are listed below. The majority were isolated from prunes (11). They are *Aspergillus amstelodami* Thom and Church (I.M.I. 17455), *A. candidus* Link, *A. carnoyi* Thom and Raper, *A. chevalieri* Thom and Church, *A. echinulatus* Thom and Church, *A. repens* de Bary, *A. ruber* Thom and Church, *A. sydowi* (Bainier and Sartory) Thom and

Church, *A. tonophilus* Ohtsuki, *Chrysosporium xerophilum* Pitt, *Sporendonema sebi* Fries, and *Xeromyces bisporus* Fraser.

Two dissimilar ascospore types were isolated in strains of *A. echinulatus*, one producing ascospores approximately 7 μm in long axis, the other 9 μm in long axis. Both isolates were studied and are designated types S and L, respectively, in this report.

**Culture media.** Cultures were grown on Czapek agar as modified by Smith (14), generally containing 20% (w/w) sucrose (pH 6.5). *A. carnoyi* required 40% sucrose; *C. xerophilum*, 40% sucrose at pH 4; and *X. bisporus*, 55% sucrose at pH 4 for optimum growth. Conidial production by members of the *A. glaucus* group was enhanced by media containing 0.5% peptone. Incubation was at 25°C, conidia requiring 2 to 4 weeks and ascospores 5 to 9 weeks to ensure maturation.

**Inocula.** Spores were dispersed by rubbing conidial chains or cleistothecia in a drop of distilled water between two cover slips. Good dispersion was achieved but with some loss of viability, presumably as a result of mechanical cell damage.

Inoculum concentrations were adjusted to give an estimated viable count of 10⁶ cells per ml, which usually corresponded to a total count of about 1.7 ± 10⁴ spores per ml.

**Medium.** So that results could be readily related to the destruction of fungi on prunes, the heating and counting medium used was a plum extract. Fresh d’Agen plums were cooked with one-third their weight of water at 70°C for 10 min, pulped, screened, treated with a pectolytic enzyme, and filtered. The pH of the extract was adjusted to 3.8, which was the mean pH of several samples of Australian d’Agen plums (11). Because xerophilic species were being studied, sucrose was added to produce a medium of 20° Brix. The water activity (aₕ) of this medium is approximately 0.98 (10). Before use, agar (2%, w/v)
was added; the medium was refiltered and pasteurized by heating at 100°C for 30 min.

**Heating.** Heating temperatures of 50, 60, 70, 75, and 80°C were used. Melted fresh plum extract medium was equilibrated to the desired temperature, and the spore inoculum was added in the proportion of 1 part of inoculum to 9 of medium, giving an initial viable count of about 10⁵ spores per ml.

At intervals, 1-ml samples were transferred from heating tubes to 5-cm petri dishes, spread by rocking, and allowed to solidify as a thin, even layer. Heating times were calculated to the moment of plating, and temperature changes during sampling were minimized by using pipettes heated to bath temperature. For initial viable counts, samples were plated in melted media at 45°C.

**Incubation.** To minimize changes in the water content of the medium, petri dishes were incubated in desiccators in which the a₀ levels were controlled by saturated solutions of appropriate salts.

The incubation period was critical: experimentation showed that the maximum viable count was obtained if incubation was terminated when the largest mycelia were 300 to 500 μm in diameter. Longer incubation periods resulted in confluent growth, whereas shorter periods reduced the number of germinations. Optimum incubation periods for most species were 45 hr to 4 days and increased with increase in heat treatment. With *C. xerophilum* and *X. bisporus*, optimum incubation periods were 2 and 4 weeks, respectively. Except for these two species, incubation was concluded after 2 weeks if growth had not occurred.

**Viable counting.** Growth was determined by adding 1 ml of lactofuchsin [0.1% acid fuchsin in lactic acid, (2)], which killed the mycelia and stained them bright red. Excess lactofuchsin was decanted from the plate after 10 min. Counts were made over the entire plate at 40 diameters magnification, by using as guidelines 2-mm squares ruled on transparent plastic under the plate. Ungerminated spores were not visible, but hyphae were readily seen if more than 15 μm in length.

**RESULTS**

The microscopical counting method proved to be an accurate but tedious technique. As many as 1,200 mycelia could be counted on a single 5-cm petri dish without confluent growth causing serious inaccuracy. Reproducibility was very high. For 52 viable counts performed in duplicate, with means between 100 and 1,200 colonies, variation from the mean averaged ± 3.2%.

**Comparative heat resistances.** The heat resistances at various temperatures of the fungi under study were compared by counting survivors after heating for 10 min. The results are shown in Tables 1 and 2, all data being based on the means of duplicate counts. Of the 16 spore types tested, ascospores of *A. chevalieri*, *A. mangini*, and *X. bisporus* were most heat-resistant, some spores from each remaining viable after 10 min at 80°C. Marked heat activation was

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Initial viable count/ml</th>
<th>Per cent survivors at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50°C</td>
</tr>
<tr>
<td><em>Aspergillus amstelodami</em></td>
<td>497</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>422</td>
<td>80</td>
</tr>
<tr>
<td><em>A. carnoyi</em> b</td>
<td>708</td>
<td>24</td>
</tr>
<tr>
<td><em>A. chevalieri</em></td>
<td>1,044</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>804</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>586</td>
<td>112</td>
</tr>
<tr>
<td><em>A. echinulatus L.</em> b</td>
<td>1,226</td>
<td>79</td>
</tr>
<tr>
<td><em>A. echinulatus S.</em> b</td>
<td>884</td>
<td>79</td>
</tr>
<tr>
<td><em>A. mangini</em> b/c</td>
<td>20 (4.0% c)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>88 (25% c)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>8 (3.5% c)</td>
<td>95</td>
</tr>
<tr>
<td><em>A. tonophilus</em></td>
<td>623</td>
<td>79</td>
</tr>
<tr>
<td><em>A. repens</em></td>
<td>869</td>
<td>76</td>
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<tr>
<td></td>
<td>469</td>
<td>93</td>
</tr>
<tr>
<td><em>A. ruber</em></td>
<td>821</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>1,525</td>
<td>95</td>
</tr>
<tr>
<td><em>Xeromyces bisporus</em> a</td>
<td>1,000</td>
<td>93</td>
</tr>
</tbody>
</table>

a Heated in fresh plum extract agar (pH 3.8), a₀ 0.98. Incubated at 25°C in atmosphere of 98% relative humidity, except as noted below.

b Incubated in atmosphere of 95% relative humidity.

c *A. mangini* showed consistently poor initial germination. Values in parentheses indicate percentage of survivors at 60°C.

d Incubated in atmosphere of 92% relative humidity.

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TABLE 1. Percentage viability of Ascospores of some xerophilic fungi heated 10 min at various temperatures
Table 2. Percentage viability of asexual spores of some xerophilic fungi heated 10 min at various temperatures

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Initial viable count/ml</th>
<th>Per cent survivors at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50°C</td>
</tr>
<tr>
<td>Aspergillus amstelodami</td>
<td>728</td>
<td>107</td>
</tr>
<tr>
<td>A. candidus</td>
<td>382</td>
<td>102</td>
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<tr>
<td>A. carnoyi</td>
<td>803</td>
<td>5.2</td>
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<tr>
<td>A. chevalieri</td>
<td>886</td>
<td>128</td>
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<td>A. ruber</td>
<td>395</td>
<td>7.8</td>
</tr>
<tr>
<td>A. sydowi</td>
<td>448</td>
<td>0.7</td>
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<td>Chrysosporum xerophilum</td>
<td>1,155</td>
<td>117</td>
</tr>
<tr>
<td>Sporendonema sebi</td>
<td>706</td>
<td>42</td>
</tr>
</tbody>
</table>

* Heated in fresh plum extract agar (pH 3.8), aw = 0.98. Incubated at 25°C in atmosphere of 98% relative humidity, except as noted below.

* Incubated in atmosphere of 95% relative humidity.

![Figure 1](http://aem.asm.org/)

**Fig. 1.** Effect of temperature on the survival of A. chevalieri ascospores at pH 3.8 and aw 0.98.

![Figure 2](http://aem.asm.org/)

**Fig. 2.** Decimal reduction times for A. chevalieri ascospores at pH 3.8 and aw 0.98.

shown by A. mangini ascospores. In experiments with plastic pouches of inoculated high-moisture prunes (9), A. chevalieri ascospores showed the highest heat resistance.

**Survivor curves.** Viable counts obtained for A. chevalieri ascospores, and corrected to an initial viable count of 10⁴, are shown as a semilogarithmic plot in Fig. 1. The data were collected from several experiments, with the age of the cultures varying from 5 to 10 weeks, which may account for some of the observed scatter. Survivor curves were constructed by a least-squares technique which constrained the curves to pass through the initial point (10⁴ viable count, zero time).

**Decimal reduction times.** From the constrained survivor curves for A. chevalieri ascospores, the following decimal reduction times were obtained: at 65°C (149°F), 50 min; at 70°C (158°F), 17.2 min; at 75°C (167°F), 6.6 min; and at 80°C (176°F), 3.3 min (Fig. 2).

By using these figures, a decimal reduction time (DRT) curve was drawn (Fig. 2). From previous work (1, 6, 18), this has been assumed to be a straight line. As recommended by Townsend, Esty, and Baselt (15), it has been drawn through the points indicating maximum heat resistance. This line is specified by a z value of 23°F and an F₁₅₀ of 2.2 min.

**DISCUSSION**

The microscopical method of viable counting used here has advantages of accuracy and reproducibility when compared with conventional plate counting and provides more information than does the simpler TDT technique. Heating and viable counting were carried out at the same aw to reproduce as closely as possible the condi-
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tions encountered by fungal spores in heat-
treated prunes. However, by taking smaller
samples from the heating suspension and mixing
with media of other a w at the time of plating,
the influence of a w on growth could be disso-
ciated readily from its effect on heat resistance.

This study has been confined to xerophilic
fungi of the types likely to cause spoilage in
inadequately heat-processed foods of relatively
low a w and pH. Within this group, the ascospores
generally have greater heat resistance than the
asexual spores of the same species. Of those
species producing asexual spores, only C. xero-
philum showed some survival after 10 min at
70 C. Among the aspergilli, the greater heat
resistance of ascospores was most marked.
This difference was the basis of the short steam
treatments used by Warcup (16) in isolating fungi
from the soil. Under appropriate conditions, iso-
lations of Ascomycetes were increased greatly
as a result of heat inactivation of most Phycy-
mycetes and Fungi Imperfecti.

Warcup (17) also found that soil samples
heated at 50 to 75 C gave much higher counts of
viable ascospores than unheated samples and
concluded that many ascospore types are acti-
vated by sublethal heating. The results obtained
with A. mangini ascospores (Table 1) show this
effect. Two isolates were used, each grown under
three sets of conditions, and in every case the
viable counts after heating at 60 C were much
greater than after heating at lower temperatures.

The scatter in viable counts of A. chevalieri
ascospores in the survivor curves of Fig. 1 can
be attributed to at least three causes: variability
in culture age, random error of sampling and
counting, and the spores' "dry" nature and con-
sequent affinity for glass and air interfaces
which reduces pipetting accuracy.

Survivor curves and DRT values have rarely
been recorded in a manner which will permit a
statistical assessment of variability (7). We
attempted this by calculating the survivor curves
by a least-squares fit, without constraint, giving
the count at zero time the same weight as counts
obtained during heating. The following DRT
values and standard deviations were obtained:
at 65 C, 45.9 ± 8.2 min; at 70 C, 19.8 ± 4.7 min;
at 75 C, 5.1 ± 1.4 min; and at 80 C, 3.44 ±
0.63 min. All of the values obtained by the con-
strained method, except that at 75 C, lay within
one standard deviation of the unconstrained
values, and it is clear that the differences be-
tween values obtained by the two methods would
not be of great practical significance.

The only previous detailed presentation of
survivor curves for fungal spores was of Penic-
cillum lapidosum Raper et Fennell (18) in which
exponential plots of survival data against time
of heating were not linear. This has been inter-
preted (12) as evidence that the exponential
rate of heat destruction commonly found for
bacteria does not apply to fungal spores. How-
ever, examination of the results (18) shows that
this conclusion is drawn from very limited data.
The curve of heat destruction of ascospores has
but three points, the first two spanning a 10,000-
fold reduction in viability. The curve for sclerotia
has a pronounced shoulder which may be ex-
plained by the finding (13) that, after several
weeks of incubation, P. lapidosum sclerotia
mature into cleistotheca, each containing
hundreds of ascospores. Each sclerotium is, in
effect, many-fold multinucleate, and a marked
depture from an exponential destruction curve
would be expected in the early stages of heating.
Although the data reported here for A. chevalieri
ascospores (Fig. 1) do show considerable scatter
and some evidence of a shoulder at two of the
four temperatures, it seems reasonable to as-
sume that the rate of heat destruction of fungal
spores is exponential.

From recently published data for the heat
resistance of X. bisporus ascospores (3), we
deduced a z value of 22 F, an F 100 of 2.0 to 2.5
min, and a DRT at 80 C of between 2.7 and 3.6
min. Our data indicate a DRT at 80 C for X.
bisporus ascospores of 3.3 min, in close agree-
ment.

The DRT curve obtained for A. chevalieri
ascospores (Fig. 2) has an extremely high z
value, 23 F (12.8 C). This z value, similar to that
deduced for X. bisporus, is higher than that usu-
ally quoted for Cladosidium botulinum (18 F;
reference 4) and much higher than those deduced
from data for P. lapidosum (18) and Byssoclamys
fulva (5) of 10.3 and 9.9 F, respectively. However,
A. chevalieri ascospores, with an F 100 of 2.2
min, are much less heat-resistant than ascospores
of B. fulva (5), with an F 100 deduced to be 50
to 100 min, depending on heating substrate, or
the sclerotia of P. lapidosum (18) with F 180 cal-
culated as 180 min.

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