Heat Resistance of *Byssochlamys* Ascospores

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Ascospores from 25 strains of *Byssochlamys* were studied for their ability to resist heat treatment in a standard defined medium. Seven of these were able to survive heating at 90°C for 25 min or longer, when initial numbers were frequently near 10⁸/ml. Ascospores from five resistant strains suspended in the medium at pH 5.0 were usually more resistant than those at pH 3.5. Rapid heat inactivation occurred for one strain at pH 6.6. Nonlogarithmic heat death rate was observed in all strains tested.

The perfect stage of the ascomycete genus *Paecilomyces* is the mold *Byssochlamys*. This organism produces eight-spored asci, the constituents of which have been shown to be resistant to routine canning processing heat (3, 7, 15). Spoilage caused by these organisms has frequently been reported in fruit and fruit juices (15, 22, 23). The incidence of *Byssochlamys* was first reported in Great Britain over 40 years ago, but subsequently investigators in many parts of the world have described their activity (19, 21–23, 28).

The circumstances affecting the ultrastructure, production, and germination of *Byssochlamys* spores have been investigated (20, 25, 31). Several researchers have looked at their behavior in heat-processed foods and described methods for detecting their presence (2, 14, 17, 21, 22). Preparation of free ascospores for a variety of studies has been developed (16).

Although there have been a number of heat resistance studies, no one has compared the behavior of *Byssochlamys* cultures derived from different geographical sources. Likewise, researchers have not tried to determine heat resistance in a standard defined medium, some of whose constituents are also ingredients in some fruit. This study was undertaken to determine the maximum heat resistance that can be expected from *Byssochlamys* under those conditions, thereby assisting processors to calculate heat treatment of their products.

**MATERIALS AND METHODS**

**Cultures.** The *Byssochlamys* strains used in this study were obtained from collections maintained by: Donald A. Corlett, Jr., The Del Monte Corp., Walnut Creek, Calif.; John J. Ellis, Northern Regional Research Center, Peoria, Ill.; Henrietta Put, Conway Laboratories, Deventer, The Netherlands; Duane T. Maundel, Continental Can Laboratories, Chicago, Ill.; and Don F. Splittstoesser, New York State Agricultural Experiment Station, Geneva, N.Y. All cultures which included strains of *Byssochlamys fulva* and *B. nivea* were maintained under refrigeration after several days of growth on unacidified potato dextrose agar slants.

**Production of ascospores.** From the beginning of these studies the need to determine an appropriate growth medium, natural or artificial, which can produce copious numbers of heat-resistant ascospores appeared important. It was observed that not all strains grew rapidly or even produced asci on what have been considered standard cultivation media such as malt extract broth and potato dextrose agar. However, it is possible that only cultures grown on natural media will produce heat-resistant ascospores such as those encountered as industrial contaminants.

Selecting a suitable spore production medium was a serious concern. In the early experiments spores were obtained by culturing the organisms on unacidified potato dextrose agar (pH 5.6) for about 30 days at 30°C. Later, in simple tests, luxuriant growth and asci formation were obtained when 2% starch, sucrose, or glucose was used in a defined broth medium containing Hoagland's base (10) and 0.04% potato infusion (24) or 0.004% potato extract (Difco), pH 4. In the method finally adopted, almost all of the *Byssochlamys* strains were cultivated in Roux flasks with the production of asci, with sucrose as the primary carbohydrate source (19). The resultant mycelium, conidiospores, and asci were homogenized in a Waring blender, filtered through two layers of sterile cheesecloth, washed twice with sterile water, and resuspended in about 40 ml of demineralized water. They were stored in a refrigerator at 5°C until used.

**Breakage of asci.** To break asci and recover single ascospores, the water suspensions were run three times through in a French pressure cell at approximately 8,000 lb/in² (16) and again refrigerated.

**Heat treatment.** A defined medium consisting of 16° Brix glucose (16 g/100 ml), 0.033 M tartaric acid (5 mg/ml), and 100 ml of demineralized water was prepared; the pH was adjusted to 3.6, 5.0, or 6.6, using 5 N NaOH, and sterilized by autoclaving (15 pounds [ca. 6.8 kg]/15 min). Suspended ascospores in the
amount of 5.0 ml were usually added to 45 ml of medium. The resulting ascospore count was dependent on the strain but was frequently near 10^6/ml, although rarely as low as 10^5/ml. Borosilicate glass thermal death time tubes preconstricted to facilitate later flame sealing were filled with 2.2 ml of inoculated medium, chilled, evacuated, and flame sealed. For the heating step, the tubes were encased in flat wire cages (1.5 by 10 by 12 cm) constructed from 0.64-cm mesh wire and immersed in a Halikainen water bath, adjusted to the test temperature, and maintained within 0.1°C.

Zero-time samples were heated separately for 30 min at 80°C (11) to stimulate activation of the ascospores. This procedure also destroyed conidiospores and hyphal fragments that were present. After each heating period four thermal death time tubes were removed and chilled in ice water. The thermal death time tubes were opened aseptically, diluted as needed in 0.15% peptone water, distributed 0.5 ml each in duplicate sterile plastic plates, and poured plated, using pH 5.6 potato dextrose agar containing 8.3 μg of rose bengal per ml. The dye concentration controlled colony spreading without affecting spore recovery (27). Plates were incubated at 30°C and counted at intervals up to 192 h.

Selecting heat-resistant strains. Preliminary tests were fashioned to select the most heat-resistant strains from those available. Ascospores from 24 B. fulva strains and 1 B. nivea strain were tested for their ability to survive heat treatment in the standard medium 16° Brix glucose tartrate (pH 3.6) after 5, 10, and 20 min of exposure at 87.8°C. Ascospores from 16 B. fulva cultures grew after treatment at the maximum heating time. The remaining nine cultures did not survive the heat treatment and were set aside. The successful strains were then subjected to similar treatment at 93.8°C. Ascospores of seven B. fulva cultures were found capable of surviving the 20-min exposure time. These were identified as: NYS-1 (Splittstoesser); M26 and M75 (Mauder); 5-6, 49-6, 50-24, and 1-24 (Corlett). A systematic study of the ascospores and heat treatment was then undertaken. For comparative purposes B. fulva 2614, a more heat-sensitive strain, was chosen from the cultures which had been set aside.

RESULTS AND DISCUSSION

Effect of pH and temperatures. The results of typical heat survival experiments are portrayed in Fig. 1. B. fulva 49-6, in a pH 3.6, 16° Brix glucose tartrate medium, produces survivor curves which exhibit a shoulder followed by an accelerating death rate. Such a curve is indicative of the often found nonlogarithmic order of death (11, 26). This type of curve was found at 80 and 87.8°C, but as the temperature was raised the log survivor curve tended to become a straight line. Ascospores from the same lot, when suspended in the same heating medium but adjusted to pH 5.0, exhibited about the same death rate characteristics as at pH 3.6. The six other B. fulva strains behaved similarly to strain 49-6.

A survivor list was constructed (Table 1) for the seven experimental strains and the heat-sensitive comparison strain, 2614. Percentage of survival after treatment at 90°C for 25 min varied over a range of 200-fold or more at both pH 3.6 and 5.0. Five of the seven experimental strains were much more heat resistant at pH 5.0 than at pH 3.6, whereas the other two and the comparison strain had approximately the same heat resistance at the two pH values. Thus, not all the strains behaved as described by Gillespy (6), who noted reduced heat resistance at the lower pH value. On the contrary, two heat-resistant strains and the comparison strain 2614 gave data in agreement with Splittstoesser et al. (26), who stated that pH had little effect on heat resistance.

Only strain NYS-1 was tested at a higher pH. At pH 6.6, it failed to survive at 90°C for 25 min (Table 1) and showed only 0.01% survival after 12.5 min (Fig. 2). Thus, there is an optimal pH for heat resistance, at least for this strain.

The pH and temperature data obtained from the seven heat-resistant strains may be compared with that of the relatively heat-sensitive B. fulva 2614, whose ascospores were not considered notably resistant in the initial arbitrary heat treatment tests. Its survival rate is much lower than that of the other seven strains (Table 1, Fig. 3). The broken survival curve at 80°C is an example of "tailing," which has been observed by many authors and reviewed by Cerf (4), who offers a number of reasons why it may be an

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Survival at:</th>
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<tbody>
<tr>
<td></td>
<td>pH 3.6</td>
</tr>
<tr>
<td>NYS-1</td>
<td>1.3</td>
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<tr>
<td>50-24</td>
<td>0.7</td>
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<td>1-24</td>
<td>0.4</td>
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<tr>
<td>49-6</td>
<td>0.4</td>
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<tr>
<td>M75</td>
<td>0.6</td>
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<tr>
<td>M26</td>
<td>7.9</td>
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<tr>
<td>5-6</td>
<td>0.04</td>
</tr>
<tr>
<td>2614</td>
<td>0.000025</td>
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artifact or may occur for other reasons such as the presence of two populations differing in heat resistance. In the heat-sensitive comparison strain, and only at a low heating temperature after a 4-log kill, this is unlikely to have practical significance.

Death rate calculations. Alderton and Snell (1) attempted to straighten death rate curves of bacterial spores so that temperature dependence calculations could be made. Their technique was most recently applied to Byssochlamys (13). The empirical expression, \[ (\log {N_0} - \log N) = kt + C \]
was adapted here to the data derived for B. fulva 49-6. The exponent \( a \) is the reciprocal of the slope of the curve plotting \( \log \) (initial count/milliliter) - (log remaining survivors/milliliter) against log time, \( k \) is a death rate constant and the slope of the linearized curve, \( C \) is the intercept, and \( t \) is the heating time in minutes. Using \( a \) for 80°C = 0.367 at pH 3.6 and 0.386 at pH 5.0, it is possible to linearize curves for all higher temperatures studied. Figure 4 is plotted from the same data as Fig. 1 as an applied illustration and is shown for comparison.

The expression used by Alderton and Snell (1) was applied to the data from eight Byssochlamys strains, and calculations representing
time in minutes to kill 10^6 and 10^7 ascospores were performed. These are summarized in Table 2. The average of calculated heating times at either pH 3.6 or 5.0 is within 2% of the observed heating times. Heat resistance was only slightly higher at pH 5.0. Comparing the values obtained by the use of Alderton and Snell's empirical expression with the observed experimental data, a correlation coefficient close to 1 at both pH 3.6 and 5.0 was achieved. Experimental 6D values could not be obtained reliably because of the relatively small initial number of spores.

Decimal reduction time values frequently are used to express the death rate of microorganisms. In this work the results of subcultured thermal death time tubes were fitted to the equation, \( D = t/(\log a - \log b) \) (ref. 18), using the logarithmic portion of the curve. \( D \) is the time in minutes required for a reduction in survivors through one log cycle, \( t \) is the heating time in minutes, \( a \) is the initial number of ascospores, and \( b \) is the number of ascospores surviving the heating time, \( t \). The estimated values expressed as 3D are presented also in Table 2. Most strains exhibited lower values when compared with the values obtained from the experimental work. Average 3D values were within 22% of the observed heating time at pH 3.6 and 42% of the observed heating time at pH 5.0. The more heat-sensitive strain 2614 exhibited the same values at both pH 3.6 and 5.0. When 3D values were compared with the observed experimental data, the correlation was very poor. Thus, if processors were to use \( D \) values as a means of determining heating times, viable ascospores of these Byssochlamys strains might still be present in the product. These data also show that the nonlinear death rate must be expressed through methods other than decimal reduction time values.

It should be noted that all of the Byssochlamys strains studied here were observed to exhibit the nonlinear rate characteristics, although at higher temperatures our graphs do not differ greatly from linearity. Hansen and Riemann (8) have reported that some investigators on heat-resistant organisms have taken log survivor curves for granted. However, deviations are frequently found (e.g., 13, 26). It has been suggested that this phenomenon occurs because there is a nonuniform distribution of heat resistance among individual cells (29).

**Thermal activation.** The methodology for heat shock in the ascospores was not optimal for all strains. Preliminary tests suggested treatment at 80°C for 30 min; however, the best exposure time and temperature varied for different strains. The plate count increased for seven strains during heat resistance trials before spore destruction ensued. B. fulva M75 exhibited this characteristic in experiments at all three test temperatures but not at all pH values studied. A full understanding of the heat activation mechanism has yet to be achieved. Several investigators have proposed that ions, buffering, and the suspending medium play an important role in developing activation enzymes (26, 27, 30, 31). The work of Eckardt and Ahrens (5) suggests that the heating temperature used here is borderline for stimulating and destroying spores and is very much dependent upon time and medium.

**LITERATURE CITED**


HEAT RESISTANCE OF BYSSOCHLAMY S ASCOSPORES


