Preparation of Free Heat-Resistant Ascospores from *Byssochlamys* Asci

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When *Byssochlamys* is grown for production of ascospores, some of the asci break up into their constituent ascospores, whereas others do not. For heat resistance studies, it is desirable to prepare a uniform suspension of free ascospores. This was accomplished with the aid of a pressure cell, from which a suspension of asci under high pressure was released to atmospheric pressure through a small orifice. Spores so treated had about the same heat resistance as untreated spores.

*Byssochlamys fulva* and *B. nivea* are heat-resistant fungi that cause spoilage in canned and bottled fruit juices and juice mixes and in some canned fruit. First noticed in England in the early 1930s, these fungi have since caused spoilage outbreaks in Europe, Africa, North America, and Australia. In the processing of fruits and juices, the heat treatment is kept mild to avoid flavor damage, and *Byssochlamys* can survive the heat treatment and grow in the finished product. It is believed to cause no health hazard; it is a matter of the esthetics (2).

These fungi are ascomycetes with spherical, eight-spored asci. The ascospores are believed to be the heat-resistant stage in the life cycle (1). Electron micrographs show a thin, structureless membrane surrounding the ascospores in the ascus (7), but the light microscope usually shows only the ascospores in their typical arrangement without any surrounding structure.

**MATERIALS AND METHODS**

**Production of asci.** The asci were produced by growing *B. fulva* strain NRRL 3493 on a thin layer of unacidified Difco potato dextrose agar for about 30 days at 30 C. The mycelium was then scraped, and the resulting mixture of hyphae and asci was suspended in water, from which the hyphae settled out so that the ascis could be decanted as previously described (3). In our experience, conidiospores are rarely if ever seen with the ascis. In this treatment, some of the asci were broken and their ascospores were released. To obtain a uniform spore preparation, it was therefore necessary to break the intact asci into ascospores or to separate them from the free ascospores.

**Spore counts.** To determine the relative numbers of asci and ascospores in a population, differential counts were made with a phase microscope at ×600. Relative frequencies of damaged and undamaged spores were determined in the same way. Dormant ascospores (either within the ascus or solitary) are refractile when seen under the phase microscope (3).

For heat resistance determinations, counts of viable asci and ascospores were made by plating on unacidified potato dextrose agar. These counts are not very precise, because *Byssochlamys* forms very thin, rapidly spreading colonies, which are difficult to see when small. As a result, we planned for about 30 colonies per plate and made several replications to partly compensate for the low numbers of colonies. Nevertheless, the precision is lower than in most plate counts of bacteria.

**Heat resistance.** Heat resistance was determined by using a thermal death-time flask (4, 5). Grape juice was preheated to 86 C and continuously stirred in a heated, three-neck flask. It was inoculated at zero time, after which samples were withdrawn at intervals for plate counts. This method is suitable for heat resistance determinations at temperatures below 100 C.

**RESULTS AND DISCUSSION**

**Preparation of free ascospores.** Asci were subjected to several treatments (Table 1, treatments 1–4) in order to break them into ascospores. None were completely successful, although treatments 2 to 4 could be used if the asci and ascospores could be subsequently separated. It is also reported that blending in a microhomogenizer (Sorvall) broke only 10% of the asci (9).

Sublethal heating with stirring in a thermal death-time flask at 86 C (the method described above for heat resistance) caused almost complete breaking of the asci into ascospores in 5 min. However, without stirring, the asci remained intact, and others have reported (9)
that asci remained intact when heated. Evidently the heat fragmentation of ascospores occurs under special conditions but is not a general phenomenon. Furthermore, ascospores heated at this temperature were heat shocked or heat damaged, and some probably were killed.

Separation of free ascospores from ascus by filtration has been reported (6). To test this method, we sonicated a suspension of ascus and passed it through a Teflon filter membrane (30-60 μm pore size; Chemware 75-X) to remove debris and then through a 75-M membrane (10-20 μm pore size). This should have been just small enough to retain the asci while passing the ascospores. In fact, although a few ascospores came through the filter, it plugged up so quickly that the yield was extremely low.

*Saccharomyces* ascus have been broken into their constituent ascospores (8) by incubation in a solution of Pronase (Calbiochem, Los Angeles, Calif.) followed by passage through a pressure cell (Aminco French pressure cell). When *Byssochlamys* ascus were suspended in water and passed through the pressure cell, 99% or more of the ascospores were liberated from the ascus (Table 1, treatment 6). This result was obtained regardless of the presence of Pronase, which was therefore omitted from further trials.

The liberated ascospores formed large aggregates that could not be dispersed by shaking. Addition of 1 or 0.1% Tween 80 to the suspension before the pressure cell treatment prevented this, except for a few small groups of two to several ascospores after some treatments.

When the treated ascospores were examined by microscopy, it was noted that some showed evidence of damage. Usually these had surface defects. A few had lost their refractivity, thus showing evidence of internal change.

| Table 1. Methods tested for releasing the ascospores from *Byssochlamys* ascus |
|-------------------------------|-------------------------------|
| Treatment | Approximate percentage of ascus remaining intact |
| 1. Shaken in bottle | 100 |
| 2. Shaken in bottle with 2-mm glass beads, 2 min | 30 |
| 3. Sonication* | 50 |
| 4. Potter-Elvehjem Teflon homogenizer, 12 passes | 70 |
| 5. Sublethal heat, stirring* | 13 |
| 6. Pressure cell (see Table 2) | <1 |

* Branson Instruments Co. model S75, 3.8 amps, 20 K, ¾-inch (about 1 cm) probe, 2 min.
* Heated for 5 min at 86°C. After 20 min of heating, only 4% of the ascus were intact.

A trial of several pressures showed that apparent damage to the ascospores was much more frequent at high pressures (Table 2). However, the plate counts were about the same throughout the pressure range, suggesting that the visually observed damage was superficial. Nevertheless, to minimize the possibility of damage, the pressure range of 4,000 to 9,000 lb/in² is recommended.

Theoretically, plate counts of the free ascospores should be almost eight times as high as those of the untreated ascus. Actually they are about four times as high. This is probably because some of the free ascospores are dormant and fail to grow in time to be counted on the plates. If germinating ascospores are observed by microscopy, it can be seen that the eight ascospores never germinate simultaneously. Thus, when they are separated and plated, some do not form colonies soon enough to be counted.

For the data shown in Table 2, samples were subjected to a flow rate out of the pressure cell of about 3 ml/min. At 9,000 lb/in², a flow rate of 20 ml/min had the same effect. At 3,800 lb/in², the 20 ml/min rate may have been somewhat less effective. The flow rate was 3 ml/min elsewhere in these experiments, although a higher rate would probably be acceptable for treatment of large amounts of material.

**Heat resistance of free ascospores.** To show that ascospores retain their heat resistance after this treatment, we compared treated and untreated spores. The free ascospores had about the same heat resistance as the ascus from which they came, as shown by a thermal death-time plot for untreated ascus and treated ascospores at 86°C (Fig. 1). Each line is the most probable (best least squares) fit to the corresponding points. That the lines have nearly the same slope shows that the treatment did not greatly change the heat resistance of the spores. As previously stated, plate counts on *Byssochlamys* lack precision, and this probably accounts for the scatter in the points.

| Table 2. Use of the pressure cell for liberation of ascospores from ascus |
|-------------------------------|-------------------------------|-------------------------------|
| Pressure (lb/in²)* | Microscopic count (%) | Plate count (×10⁴) |
| | Damaged spores | Intact spores | Intact ascus |
| 15,000 | 40 | 60 | 0 | 270 |
| 11,500 | 19 | 80 | 1 | 260 |
| 9,000 | 16 | 84 | 0 | 280 |
| 6,400 | 2 | 98 | 0 | 260 |
| 3,800 | 2 | 98 | 0 | 240 |
| Control | 0 | 11 | 89 | 68 |

* Flow rate of 3 ml/min.
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That the lines almost coincide is consistent with the fact, previously stated (Table 1, treatment 5), that the heat and stirring break up the untreated asci, so that the counts reflect the number of individual free ascospores even in the untreated control.

The D values calculated from these lines are, respectively, 13 and 14 min for the control and treated ascospores. (The D value is the time, at a given temperature, after which only 10% of the spores survive.) This can be compared with a D value of 16 min at this temperature which we obtained several years ago for asci of the same strain of Byssochlamys by using thermal death-time cans (3). Considering that the method, the spore crop, and the grape juice were all different, the two sets of data are in reasonable agreement.

Thus we have shown that, by using a pressure cell as described here, it is possible to prepare a suspension of free Byssochlamys ascospores that have not been heat shocked or significantly damaged. They have about the same heat resistance as if they had remained in the intact asci up to the time of the heating experiment.

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