Enumeration of *Byssochlamys* and Other Heat-Resistant Molds

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Methods for the detection of low numbers of heat-resistant molds on fruits were studied by using cultures of *Byssochlamys* and a number of unidentified mold isolates. Ascospore dormancy had a marked effect on viable recoveries, and the medium in which ascospores were heated influenced activation rates. Best results were obtained when fruit homogenates were heated for 60 min at 70 C in Concord grape juice, followed by culturing on acidified Potato Dextrose Agar.

In recent years, there has been an increase in the spoilage of thermally processed fruit products caused by *Byssochlamys* and related molds (6). The problem is that their ascospores are able to survive the fill temperatures of 80 to 90 C commonly used as the process for these foods. Increasing the severity of the thermal treatment is not a solution because too often this results in a marked reduction in product quality. It appears that the best method for preventing spoilage is to control contamination. This, in turn, requires sensitive methods for the detection of viable spores on fruit and in processing lines.

Numerous methods have been used in culturing for *Byssochlamys*. It has long been known that the ascospores exhibit a dormancy that can be broken with heat (3), and a variety of treatments, ranging from 5 min at 75 C to 35 min at 80 C, have been used (2, 5, 7, 11). After this, the material usually has been cultured on a nutrient medium such as potato-sucrose or potato-dextrose-agar. Growth of bacterial spores that would survive the heat shock has been prevented by acidifying the agar (4) or by the inclusion of compounds such as chloramphenicol (9) or hexachlorophene (1). Little information is available regarding the effectiveness of the different activation and cultural procedures.

MATERIALS AND METHODS

**Cultures.** The named cultures of *B. fulva* and *B. nivea* were obtained from J. J. Ellis of the Northern Regional Research Laboratory, U.S. Department of Agriculture. The others were isolated in this laboratory from spoiled fruit product and from samples collected from nearby orchards and vineyards.

**Ascospore production.** Ascospores were obtained from cultures grown as a pellicle over broth. Two media, 5% Difco Malt Extract Broth and Concord grape juice, have been used. The latter was prepared by adjusting the concentration of commercial grape concentrate to 15 C Brix as measured with a hand refractometer.

When only small quantities of spores were needed, a typical trial consisted of inoculating 5 ml of broth in a 16- by 150-mm culture tube (10). After incubation for 28 days at 32 C, the mat and media were transferred to a sterile 50-ml chamber for blending in a Sorvall Omni-mixer homogenizer. After a visually homogeneous suspension was obtained, the material was centrifuged and washed three times in sterile distilled water. Suspensions to be used in later experiments were frozen and stored at -23 C.

**Activation.** In most trials, 0.5 ml of washed spores was added to 4.5 ml of the activation medium. The tube then was placed in a water bath for the desired time period. The media formulas represent concentrations after dilution by the spore suspensions.

To assure that different responses were not artifacts resulting from clumping or the breaking up of asci, microscopic counts, determined with a hemocytometer, of single spores and asci were conducted routinely before and after treatment.

**Viable counts.** Appropriate dilutions of spore suspensions were cultured on Difco Potato Dextrose Agar acidified to pH 3.5 with tartaric acid. The incubation was at 32 C for 2 to 4 days depending upon the strain.

RESULTS AND DISCUSSION

**Heat activation.** The heat-labile structures present in culture homogenates, principally conidia and hyphal fragments, may mask much of the effect of heat on ascospores. Heating a homog-
enate at 70 C, for example, reduced the viable count by over 3 log cycles during the first 5 min, followed by a slight increase in count due to activation of dormant ascospores (10). The kinetics of activation during the initial 5 min could not, of course, be measured.

Attempts to obtain pure suspensions for these studies by repeated centrifugation and by filtration (8) were not successful. Although asci could be concentrated by slow, short-time centrifugation, the method was tedious and the preparations were never completely free from conidia and hyphae. The filtration technique was not applicable because of the large numbers of conidia produced by certain strains.

Fortunately, it was found that suspending homogenates in 85% ethanol for a short time destroyed the conidia and hyphae (Table 1). Heat and ethanol both reduced the viable count of NYS 1, composed mainly of conidia, by about 4 log cycles, indicating that the two treatments inactivated the same structures. With NRRL 2614, mainly asci, the counts obtained with the "ethanol only" and "washed only" (control) treatments reflected the low populations of active asci that were present. Heating these suspensions increased this population about 10-fold. It was concluded that ethanol did not activate the ascospores or affect their viability, and, therefore, the treatment was used routinely.

Early studies on the effect of the heating menstruum on activation included grape juice because it represented one of the foods in which a methodology for spore detection was desired. The results of numerous trials showed an interaction between the medium and temperature.

| Table 1. Effect of ethanol and heat on the viable population of mold homogenates |
|----------------------------------------|-------------------------------|
| Homogenate | Treatment before plating | Count/ml |
| NYS 1 (mainly conidia) | Ethanol, heateda | 22 \times 10^4 |
| | Heated only | 26 \times 10^4 |
| | Ethanol only | 11 \times 10^4 |
| | Washed only | 18 \times 10^4 |
| NRRL 2614 (mainly asci) | Ethanol, heateda | 11 \times 10^4 |
| | Heated only | 12 \times 10^4 |
| | Ethanol only | 10 \times 10^4 |
| | Washed only | 6 \times 10^4 |

a Homogenates were suspended in 85% (final concentration) ethanol for 20 min, washed two times, resuspended in water, and then heated at 70 C for 60 min before plating on acidified Potato Dextrose Agar.

When spores in Concord grape juice were heated at 60 C, activation was completed in 60 min or less, whereas heating in water for as long as 3 hr produced no detectable increase in the viable count (Fig. 1). Spore activation also was enhanced by grape juice at 70 C, although at this temperature a significant number of the spores in water now were activated. These data indicated that grape juice accelerated the rate of spore activation.

The spores from numerous strains have been activated at a variety of temperatures in grape juice. At 40 C, the lowest temperature studied, a slight increase in viable count was detected after heating for 3 hr, although 95% of the activatable spores remained dormant. At 80 C, the counts usually were lower than at 70 C, indicating that this temperature was lethal for the spores of many strains. It was concluded from this that 70 C was the optimal activation temperature for most strains. However, a lower temperature such as 60 C was more suitable for studying certain variables, because it emphasized differences in activation rates.

The stimulatory effect of grape juice varied with the variety, with Concord being more active than juice from Riesling and Seibel 9549 grapes (Fig. 2). Other trials supported these data in that...
Concord juice could be diluted to a concentration as low as 2° Brix without reducing activation rates, whereas any dilution of Seibel 9549 juice below the original 16° Brix resulted in fewer spores being activated.

Studies on the mechanism of grape juice stimulation revealed an interaction between pH and the active factor (Fig. 3). Adjusting Concord juice to pH 4 caused a marked reduction in the number of spores activated, whereas at pH 5 to 7, the viable counts were no higher than obtained in water. It is known that pH per se was not responsible for the grape juice effect, because heating spores in pH 3.5 solutions of malic and tartaric acids, the principle acids in grape juice, failed to enhance activation (Table 2).

The data (Table 2) also show that, although glucose and malt extract broth had no effect, a 5% solution of yeast extract gave results comparable to Concord juice. It appears that the same principle was active in both menstrua since their activity responded similarly to changes in pH.

Other trials have shown that the factor was still present after grape juice was dried at 100 C, autoclaved, or fermented. Work presently is underway to isolate and define the compound.

Cultural conditions. At first, most-probable-number (MPN) procedures employing broth cultures were used to enumerate ascospores on fruit samples. It was assumed that the incidence of heat-resistant spores would be low, and, therefore, the method would have the advantage that it permitted relatively large samples, 25 g or more, to be cultured. Unfortunately, the method was found to require a very long incubation period. In one trial, for example, 50-ml amounts of 5% malt broth in each of 10 milk dilution bottles were inoculated with 10 g of grape homogenate containing an average of two active asci. The bottles were incubated at 32 C, on their sides with caps loosened to facilitate aeration. The first growth, in one bottle only, was detected after 7 days, whereas 22 days was required before all of the potentially positive bottles, a total of nine, showed growth. Although the MPN recovery figures were in good agreement with the number of asci in the inoculum, the long incubation reduced the effectiveness of the method.

When inoculated homogenates were cultured concurrently in broth and on agar media, the latter were found to yield maximal viable recoveries after an incubation of only 2 to 4 days at 32 C. More rapid growth because of better aeration plus the fact that colonies were more...
Table 2. Spore activation in various menstrua

| Strain                    | Activation diluenta | Ratio of viable count (diluent/water activated)b |
|---------------------------|--------------------|--------------------------------------------------|
| Isolate F-2               | Concord grape, 16° Brix  5% Difco yeast extract, pH 3.6  5% Difco Malt Extract Broth, pH 3.6 1% Tartaric acid, pH 3.5 Ash, Concord juice, pH 3.5 | 23 22 4 2 1 |
| *Byssochlamys fulva* NRRL 2614 | Concord grape, 16° Brix  5% Difco yeast extract, pH 3.6  5% Difco yeast extract, pH 6.6 15% Glucose, pH 3.6 | 71 70 1 1 |
| *B. nivea* NRRL 2260      | Concord grape, 16° Brix  0.1% Malic acid, pH 3.7 | 50 0.5 |

a The pH of low-acid diluents was adjusted with tartaric acid.

b Activated at 60°C for 60 min before plating on acidified Potato Dextrose Agar.

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FIG. 4. Procedure adopted for the detection and enumeration of heat-resistant mold spores. *PDA, Potato Dextrose Agar.*

easily seen on agar probably accounted for these results.

The following agar media have been compared to determine which gave the higher viable counts when inoculated with activated ascospores: Potato Dextrose, Plate Count, 5% yeast extract, 5% Malt Extract, Put’s (9), and concentrations of Concord grape juice from 1 to 15° Brix. The results indicated that many heat-resistant molds were not particularly fastidious in that a number of strains gave comparable counts in all media. When differences were noted, acidified Potato Dextrose Agar usually provided the higher figures. For example, a study of 11 strains showed that 5 gave higher counts in this medium than in 7.5°-Brix grape juice-agar. In general, the differences were by a factor of twofold or less which indicated that the plating medium was not as important a variable as was the heat activation menstruum.

Adopted methods. Figure 4 illustrates the procedures used for the detection and enumeration of low numbers of heat-resistant molds on a variety of fruit samples. To minimize the opportunity for chance contamination, a potential problem in laboratories in which *Byssochlamys* is routinely cultured, the sterile, screw-cap blender jars were usually carried to the sampling site. Although the amounts varied, 50 to 100 g of fruit often was blended with 100 ml of Concord juice. A 5-min treatment usually produced a homogeneous mixture that could be readily poured into petri dishes.

Heat activation was carried out in the blender jars. The jars were enclosed in polyethylene bags before being placed in the water bath as a safeguard against leakage through the bottom bushing. A 2-hr hold assured that the contents were at the equilibrium temperature for about 1 hr. Overheating was not a problem since spores have been held for as long as 6 hr at 70°C without reducing the viable count. After heating, the entire contents of the jar were distributed into petri dishes, approximately 10 ml per dish. Equal volumes of double-strength Potato Dextrose Agar then were added to the plates. The culturing of such a large sample was required because usually the level of spore contamination was very low. The method underestimated spore populations to some extent because of material retained on the blender walls. Most strains produced countable colonies by 48 hr at 32°C. Because a few required
a longer incubation, negative plates were held for 96 hr before being discarded.

The above method has been used for the examination of over 60 samples collected from orchards, vineyards, and processing lines. The procedure appears to be effective in that many contaminated samples, ranging in spore counts from under 1 to over 1,000 per 100 g of grapes, have been detected and some 40 different mold types have been isolated. Interference by other microorganisms has not been a problem. These microorganisms apparently were eliminated by heating at 70 C coupled with the low pH plating medium.

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