Novel Technique for Isolating Microstructures Present in Shake Cultures of the Fungus Ceratocystis ulmi

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Microstructures found in shake cultures of Ceratocystis ulmi, the fungus causing Dutch elm disease, have been isolated by a novel technique using the effect of bubbling gas through the culture filtrate.

During routine observations of shake cultures of our strain of Ceratocystis ulmi (Buism.) C. Moreau, small particles (microstructures) were periodically noticed. Ouellette and Gagnon (2) also reported small particles in their strain, which they termed microendospores and microhyphae; Érottzmann and Campana (1) pointed out experimentally that these were not viable. In order to characterize the particles observed in our cultures, an isolation technique was necessary. Techniques involving centrifugation and solvent extraction were unsuccessful. However, the supposition that these particles may be associated with gas resulted in the development of a technique which was successful and is described in this report.

A strain (CESS-16K) of the fungus C. ulmi was cultured in Wilson modified medium in which 1% sucrose was used as the carbon source. The cultures (25 ml each) were kept at 23°C on a gyratory shaker (120 rpm). After 10 days, the cultures were removed from the shaker and centrifuged for 30 min at 1,000 x g to eliminate precipitable solid material including fungus spores and hyphae. After centrifugation the supernatant fluid was slowly decanted. It was then filter-sterilized through a series of Milipore membrane filters with pore diameters of 0.8, 0.45, and 0.22 μm, respectively, in order to remove any additional small spores and hyphae not precipitated by centrifugation. The 0.22-μm filtrate was then divided into 75-ml samples.

The apparatus used for the isolation of microstructures is illustrated in Fig. 1. The primary part (A) of the apparatus consists of a capsule constructed of two 50-ml polycarbonate centrifuge tubes, fused together by the solvent, 1,2-dichloroethane. Holes (0.5 inch, approximately 1.25 cm) were drilled through each end. The fritted-glass cylinder portion (medium porosity) of a gas-dispersion tube was inserted into the capsule through the bottom hole and held in a vertical position by a punched rubber serum stopper. The stopper served not only as a holder but also as a seal. A 0.25-inch (approximately 0.63-cm) hole was also drilled through the capsule wall 0.5 inch above that level marked for 75-ml filtrate.

The secondary part (B) of the apparatus consists of a retainer (50-ml polycarbonate centrifuge tube) and a two-hole rubber stopper supporting two tubes, one of which has a tapered end. The tube with the tapered end was used to connect the retainer to the capsule. The tapered portion was pushed through the hole previously mentioned, and the tip was positioned to the center of the capsule. The other tube was used to connect the retainer to a water-aspirator line (vacuum).

The filtrate (75 ml) was poured slowly into the capsule through the top hole by means of a funnel. N2 gas was then applied at 1 lb/in². As the N2 passed through the fritted-glass cylinder, it entered the filtrate as small bubbles. Bubbling resulted in the accumulation of white, foamy material on the top of the filtrate. Small bubbles worked better than large ones. A vacuum was then applied to suck this material through the tube with the tapered end and deposit it in the retainer. The procedure above was continued until the foamy material could no longer be collected. This material consisted of the microstructures originally set out to be isolated.

Characteristics which were observed for these microstructures are shown in Fig. 2. The bub-
Bubbling causes the "unit" structures first seen in cultures of *C. ulmi* to assemble gradually into "rods" and "fibrils" which accumulate on the filtrate surface. As this occurs, the clear filtrate becomes milky or turbid in appearance. By centrifugation, the assembled structures disassemble and revert to "unit" structures, and the filtrate becomes clear once more. Reassembling can be achieved by bubbling. However, when detergent was added (sodium deoxycholate at a final concentration of 0.5%), the "rods" and "fibrils" formations were disassembled into "unit" structures. The fact that bubbling at this time had no effect suggests the possible elimination of the means by which bubbling causes the "unit" structures to assemble into "rods" and "fibrils".

From each 75 ml of filtrate, a sample of 1 to 2 ml can be obtained by the bubbling technique. Microscope examination of the isolated sample revealed numerous "rod" and "fibril" formations and no spores and hyphae. The characteristics of this sample were the same as those outlined above; hence, these were the correct microstructures. According to the preliminary tests, these microstructures are presumed not to be viable.

In order to isolate these microstructures by means of the bubbling technique, the ability of "unit" structures to assemble into "rods" and "fibrils" appears essential. It is suggested that if any material of this nature is present in liquid systems, the bubbling technique could be a method for isolation. The bubbling technique brings out a specific characteristic of these microstructures which is being used at present to isolate an amount sufficient for further purification and characterization, so that studies may be carried out on their possible significance in the development of Dutch elm disease.

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**LITERATURE CITED**

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