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Specimen Holder to Critical-Point Dry Microorganisms for Scanning Electron Microscopy

C. P. KURTZMAN, F. L. BAKER, AND M. J. SMILEY

Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

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Critical-point drying of microorganisms for scanning electron microscopy can be rapidly and effectively accomplished by use of a newly described specimen holder. Up to eight different samples of spores or vegetative cells are placed between polycarbonate membrane filters in the holder and processed through solvent dehydration and critical-point drying using carbon dioxide without loss or cross contamination of microorganisms. Yeasts, molds, bacteria, and actinomycetes have been successfully processed.

The scanning electron microscope (SEM) is being used with increasing frequency to study microorganisms. Although cell collapse is a major problem encountered in specimen preparation, collapse can be prevented by such methods as critical-point drying (1, 8), freeze drying (10), or quick freezing followed by observation on an ultra-cold stage (5).

Of these methods, critical-point drying is perhaps most commonly used. It involves solvent dehydration of the specimen and replacement of the dehydrating solvent with a second solvent (intermediate fluid) that must be miscible with the liquified gas (transitional fluid) used to dry the specimen of solvent. Techniques are available for using carbon dioxide (1, 8), certain types of Freon (3), or nitrous oxide (7) as the transitional fluid. Cells must be dehydrated in graded solvent systems, such as ethanol or acetone, before processing with either Freon or carbon dioxide. Koller and Bernhard (7) reported dehydration was not required when using nitrous oxide, but other investigators have had no success with the nitrous-oxide technique (2, 8). Lewis and Nemanic (8) give the rationale for critical-point drying along with information on the necessary methodology.

Macroscopic tissue samples can be easily processed for critical-point drying, but numerous handling problems occur with microorganisms. These include loss of cells and clumping of the cells from contact with solvents. Talens et al. (11) reported confining yeast cells between pieces of filter paper; Nemanic (9) held protozoa in a perforated Beem capsule.

We have developed a convenient and effective procedure to prevent loss of cells by holding microorganisms between two 0.4 μm or 1.0-μm Nuclepore filters while they are carried through all steps of critical-point drying. This method has been used for ascospores and vegetative cells of several yeast genera and also has been successfully tested on selected bacteria, actinomycetes, and the fragile vegetative spores of certain filamentous fungi.

MATERIALS AND METHODS

Organisms and culture conditions. All microorganisms used in this work are maintained in the Agricultural Research Service Culture Collection at the Northern Laboratory. Much of the developmental work was done with ascospores and vegetative cells of *Saccharomycopsis vini* (Kreger-van Rij) van der Walt et Scott NRRL Y-7289 and *Debaryomyces vanriji* (van der Walt et Tscheuschner) Abadie et al. NRRL Y-7431. Both were grown on yeast-malt agar at 25 and 15 C, respectively. Other species observed include *Bacillus cereus* var. *thuringiensis* Smith et al. NRRL B-4039 (glucose, yeast extract, inorganic salts medium, 28 C), *Streptomyces diastaticus* (Krainsky) *sensu* Pridham et al. 1965, NRRL B-5765 (glucose, yeast extract, inorganic salts agar, 28 C), *S. griseus* (Krainsky) Waksman et Henrici NRRL B-5766 (inorganic salts, starch agar, 28 C), and *Rhizopus* sp. NRRL 5857 (potato-glucose agar, 25 C).

Filters. DeNee and Stein (4) and Johari and DeNee (6) discussed the suitability of Gelman, Millipore, Flotronics (fritted silver), and Nuclepore filters for collection and observation of small particles by SEM. Of these four, Nuclepore filters were judged most suitable because of their smooth, flat background. Because of their resistance to the organic solvents used in critical-point drying, we tested both Nuclepore (made of a special polycarbonate, General Electric, Irradiation Processing Center, Vallecitos Nuclear Center, Pleasanton, Calif.) and fritted silver filters (Selas Flotronics, Spring House, Pa.). Nuclepore filters of 0.4-μm and 1.0-μm pore size were tested uncoated and vacuum coated with either

gold-palladium alloy (60:40), aluminum, or carbon at thicknesses of approximately 15 and 25 nm, whereas fritted silver filters of 0.2-μm and 1.2-μm pore size were used uncoated. Nuclepore filters gave a more uniform background and were used exclusively in this study. Filters 13 mm in diameter are the best size because they fit on SEM specimen stages (stubs) without trimming. However, because of ease in handling during coating, 47-mm diameter Nuclepore filters were used, and after coating seven to eight 13-mm diameter disks were cut from each of them with a cork borer.

Culture preparation. Yeast ascospores were freed from asci by digestion at 25 C with the enzyme preparation Glusulase (Endo Laboratories, Inc., Garden City, N.Y.). The enzyme-spore mixture was added to 5 ml of a 0.3% solution of the laboratory detergent Haemo-Sol (Haemo-Sol, Inc., Baltimore, Md.) and the spores were separated by low-speed centrifugation. The spores were washed two additional times in this manner, and the final volume was adjusted to 0.2 ml by decanting.

A 13-mm diameter filter was then placed over the support-containing half of a Swinnex filter unit (Millipore Corp., Bedford, Mass.), and a small drop of the spore suspension was spread over the surface. Another filter was placed over the spread spore suspension and the filter unit was assembled. The filter unit was attached to a syringe and the ascospores were washed first with 10 ml of 0.3% Haemo-Sol and then with 10 ml of distilled water. After removal from the filter unit, the filters were kept together and placed in the specimen holder illustrated in Fig. 1. The size of the holder, and consequently the number of filters that can be accommodated, is dictated by the size of the pressure bomb available for critical-point drying. When more than one sample was being prepared, the filters were stored on moist filter paper in a petri dish to keep them from drying out; then all were loaded into the holder at the same time. Both the Haemo-Sol solution and the distilled water were passed through a 0.45-μm Millipore filter before use to remove debris.

The rigorous washing steps outlined were usually only necessary for ascospores freed enzymatically. Free spores, yeast vegetative cells, and cells of other microorganisms were washed in centrifuge tubes three times with Haemo-Sol solution, followed by one 5-ml distilled water wash, and then were placed between filters and transferred directly to the specimen holder.

Fixatives. Spores and vegetative cells of *S. vini* and *D. vanriji* were fixed in 3% glutaraldehyde for 2 h at 25 C followed by 1% osmium tetroxide for 16 h at 5 C. Each fixative was also tested alone. Both fixatives were made up in 0.05 M sodium phosphate buffer (pH 7.0). Since fixation did not improve the image quality of these two species, fixatives were omitted in subsequent work.

Dehydration. Graded ethanol (10, 30, 50, 95, 100%) and acetone (30, 50, 70, three changes 100%) series were tested for dehydration with cells held between filters in the assembled specimen holder. The holder was passed through each series at 10-min intervals. After passage through the ethanol series, the holder was placed in the intermediate fluid amyl acetate for 10 min. Wide-mouth screw-cap bottles of 65-ml capacity were convenient for holding the dehydrating reagents. The holder greatly simplified dehydration because higher solvent concentrations cause clumping of cells that leads to considerable handling difficulties when dehydration is carried out in centrifuge tubes.

Critical-point drying. After dehydration, the specimen holder was quickly placed in the pressure bomb of a Denton DCP-1 critical-point drying apparatus (Denton Vacuum, Inc., Cherry Hill, N.J.) which was slowly flushed with liquid carbon dioxide at approximately 900 lb/in^2 for 50 min. After this treatment, the bomb was sealed and heated in a 50-C water bath until maximal pressure was reached, which must exceed 1,072 lb/in^2; then the pressure was slowly released over a period of 30 to 40 min. After removal of the filter assemblies from the holder, the upper filter was peeled off and discarded. The lower filter was cemented at the periphery to a SEM stub with silver electrical conductive paint and vacuum coated to a thickness of about 15 nm with gold-palladium alloy. Filters must be cemented with rather thick conductive paint; otherwise, the paint solvent will flow across the filter and ruin the cell preparation.

The preparations were examined in a Cambridge Stereoscan Mark II scanning electron microscope at an accelerating voltage of 20 kV; the final aperture was 200 μm, and the specimen stage was inclined 45° to the incident beam.

RESULTS AND DISCUSSION

The specimen holder and procedures devised for critical-point drying microorganisms are illustrated in Fig. 1. Cleanliness of spores and vegetative cells is of utmost importance and will vary depending on the species and culture conditions. Yeast ascospores enzymatically freed from asci need washing with 0.3% Haemo-Sol or other detergent rather than distilled water. Washing in centrifuge tubes is sometimes satisfactory, but often further washing in a Swinnex filter unit is mandatory for a clean preparation. However, washing in centrifuge tubes with 0.3% Haemo-Sol, followed by distilled water, was nearly always satisfactory for yeast vegetative cells and the other microorganisms tested. Relatively clean material, such as aerial fungus conidia, might simply be suspended in a drop of distilled water and directly applied to the filter.

Ethanol dehydration of microorganisms or tissues, followed by infiltration with amyl acetate, has given satisfactory results in a wide variety of previous studies, but because of their physical-chemical characteristics, these liquids penetrate the filters rather poorly and many cells are collapsed after critical-point drying. This problem was circumvented by use of an acetone dehydration series, and since acetone also functions as an intermediate fluid amyl

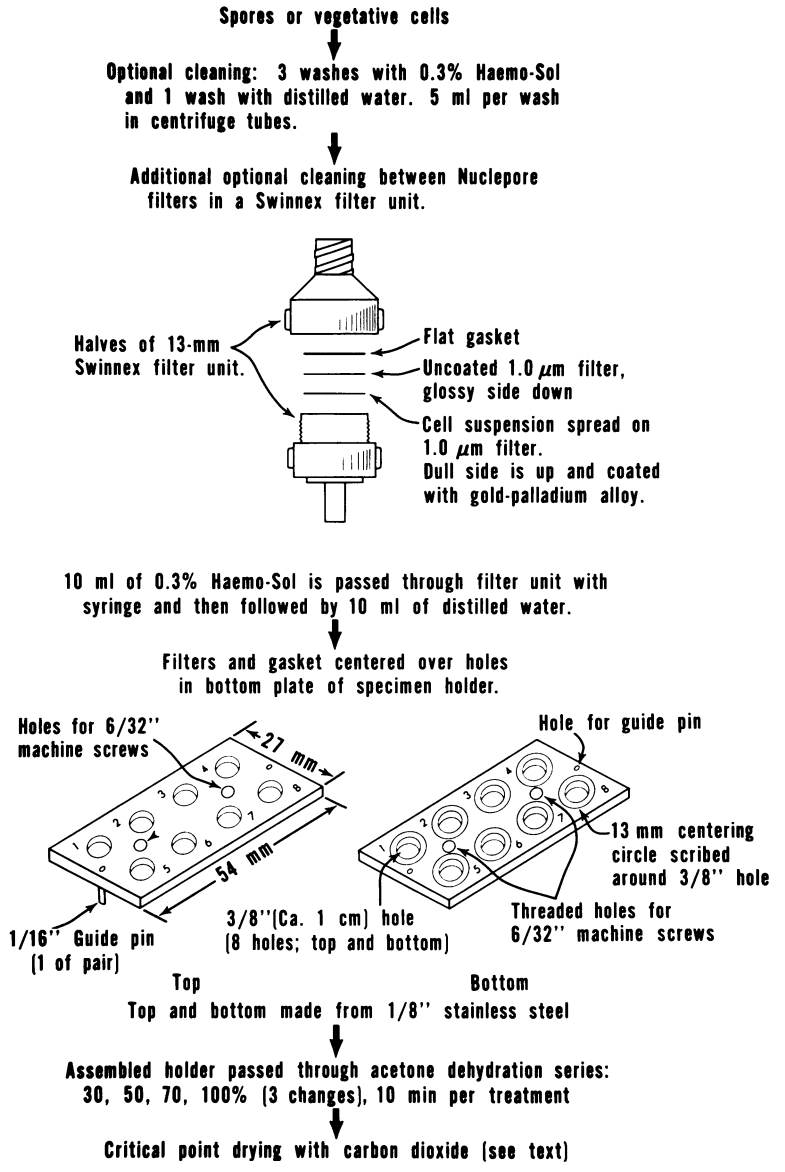

FIG. 1. *Specimen holder and procedures used for critical-point drying.*

acetate was not needed.

After critical-point drying, the Nuclepore filter holding the cell suspension is mounted on an SEM stub and vacuum coated with 15 nm of gold-palladium alloy. A beam current of 20 kV charged the filter excessively. Precoating the filters with 15 nm of gold-palladium alloy before use prevented this charging, and then, after processing, the usual gold-palladium coating was applied to the cell preparation. Precoating filters with about 15 nm of carbon before use was also satisfactory. Doubling or tripling the thickness of the precoating on 1-μm filters resulted in some collapse; this indicated poor penetration of processing liquids. Even the 15-nm precoating on 0.4-μm Nuclepore filters also seemed responsible for some collapse. We found that uncoated filters of either pore size could be successfully used if, after processing, a 15-nm carbon coating was applied before the final gold-palladium coating. This finding is noteworthy if an investigator must use filters having 0.4-μm pores or if the microorganism is grown on the filter surface before processing.

Nuclepore filters have a smooth and a dull side. Both sides give a satisfactory image, but the dull side allows the specimen suspension to be spread evenly over the filter.

Substituting the coated filter with a circular cover glass or a disk of aluminum foil frequently gave preparations with many collapsed cells. Apparently solvent penetration through a filter on only one side of the cell preparation is inadequate. A generally satisfactory alternative to viewing the preparation on filters is to transfer the cells to a glass surface. Glass squares (8 by 8 mm) cut from microscope slides were cemented to SEM stubs and a drop of double-sticky cellophane tape adhesive dissolved in acetone [1 square inch (about 2.54 cm squared) of tape in 20 ml of acetone (6)] was spread over the surface of the glass and allowed to dry. The stub was then momentarily pressed against the surface of a filter to remove the cells.

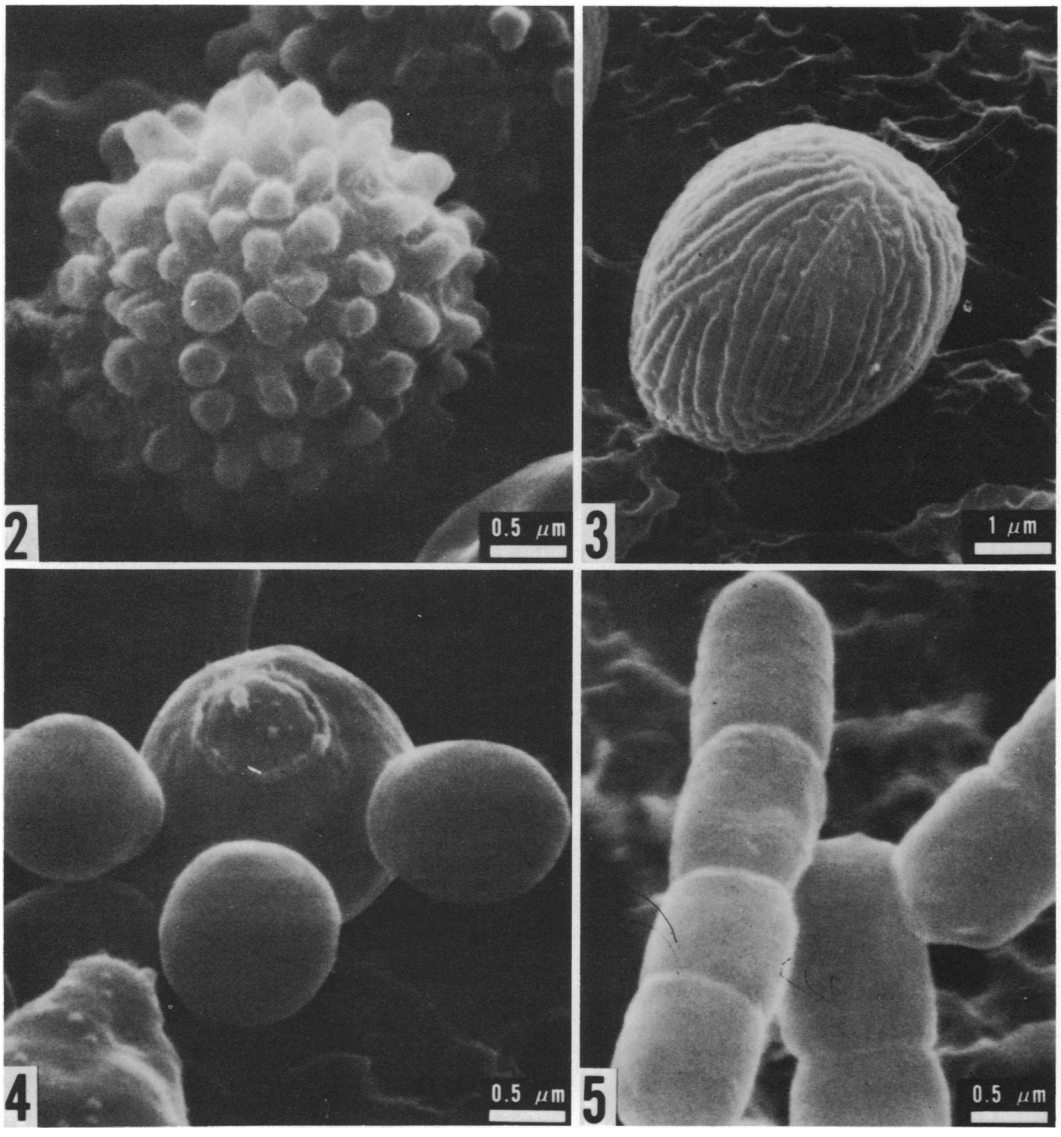

FIG. 2. *Ascospore of Debaryomyces cantarellii NRRL Y-7421.*
FIG. 3. *Sporangiospore of Rhizopus sp. NRRL 5857.*
FIG. 4. *Budding cell of Debaryomyces vanriji NRRL Y-7431.*
FIG. 5. *Vegetative cells of Bacillus cereus var. thuringiensis NRRL B-4039.*

These preparations were also coated with gold-palladium alloy.

Results were uniformly good with a variety of microorganisms that were critical-point dried in the specimen holder. Selected species, all of which show considerable collapse without critical-point drying, are illustrated in Fig. 2 through 5 and were processed between 1.0-μm Nuclepore filters coated with gold-palladium alloy. *Streptomyces diastaticus* and *S. griseus* are other species that also preserved well.

Although some microorganisms apparently do not collapse without special processing for SEM, the majority do collapse, and some preservative method such as critical-point drying is necessary for realizing the greatest amount of information. The specimen holder and procedures reported here allow as many as eight microorganisms to be processed through critical-point drying at one time with rapidity and freedom from cross contamination.

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