Biomédica 2003;23:225-31

TECHNICAL NOTE

Comparative effect of osmium tetroxide and ruthenium tetroxide on *Penicillium* sp. hyphae and *Saccharomyces cerevisiae* fungal cell wall ultrastructure

Orlando Torres-Fernández [1], Nelly Ordóñez [2]

[1] Laboratorio de Microscopía Electrónica, Instituto Nacional de Salud, Bogotá, D.C., Colombia.
[2] Laboratorio de Patología, Instituto Nacional de Salud, Bogotá, D.C., Colombia.

The fungal cell wall viewed through the electron microscope appears transparent when fixed by the conventional osmium tetroxide method. However, ruthenium tetroxide post-fixing has revealed new details in the ultrastructure of *Penicillium* sp. hyphae and *Saccharomyces cerevisiae* yeast. Most significant was the demonstration of two or three opaque diverse electron dense layers on the cell wall of each species. Two additional features were detected. *Penicillium* septa presented a three-layered appearance and budding *S. cerevisiae* yeast cell walls showed inner filiform cell wall protrusions into the cytoplasm. The combined use of osmium tetroxide and ruthenium tetroxide is recommended for post-fixing in electron microscopy studies of fungi.

Key words: fungal cell wall, fungal ultrastructure, osmium tetroxide, ruthenium tetroxide, *Penicillium* sp., *Saccharomyces cerevisiae.*

Efecto comparativo del tetróxido de osmio y del tetróxido de rutenio como fijadores sobre la ultraestructura de la pared celular de hongos

Al microscopio electrónico, la pared celular de los hongos es de apariencia translúcida en especímenes procesados mediante la posfijación convencional con tetróxido de osmio. La posfijación con tetróxido de rutenio reveló nuevos detalles ultraestructurales en hifas de *Penicillium* sp. y en levaduras de *Saccharomyces cerevisiae.* El aspecto más destacado fue la modificación en la transparencia de la pared celular, característica de la fijación con tetróxido de osmio. El tetróxido de rutenio permite verla como una estructura compuesta por dos o tres capas oscuras de diferente tonalidad. Se observaron también otros dos rasgos importantes: los septos en las hifas de *Penicillium* sp. presentaban una apariencia trilaminar y en la pared celular de las levaduras de *S. cerevisiae*, en etapa de gemación, se destacaban unas prolongaciones filiformes que emergían desde la capa interna y se proyectaban hacia el citoplasma. Se recomienda el uso combinado de los dos fijadores en estudios ultraestructurales de los hongos.

Palabras clave: pared celular, ultraestructura de los hongos, tetróxido de osmio, tetróxido de rutenio, *Penicillium* sp., *Saccharomyces cerevisiae*.

The two-step fixing procedure, glutaraldehyde followed by osmium tetroxide (OsO_4) (1), continues to be the preferred method for preserving fine structures in biological samples for electron microscopy studies. Although ruthenium tetroxide (RuO_4) was proven effective as an alternative post-fixative (2), this reagent decomposes rapidly and has poor tissue penetration (3,4). However, RuO_4 is an excellent fixative for revealing the existence of some non-OsO_4-fixed cellular compounds. RuO_4 has been used mainly to visualize extracellular saturated lipids in ultrastructural studies of stratum corneum from skin. These lipids constitute the dermal permeability barrier (5,6).

The ability of RuO_4 to preserve previously unknown ultrastructural details in microsporidian (Protozoa) spores has been demonstrated recently. The

Correspondencia:
Orlando Torres-Fernández, Avenida calle 26 No. 51-60, Bogotá, D.C., Colombia
otorresf@hemagogus.ins.gov.co

Recibido: 20/11/02; aceptado: 23/05/03

microsporidian spore wall consists of a chitin-protein complex compound with saturated lipids probably embedded in it (7,8). Microsporidia and fungi are structurally and phylogenetically related microorganisms (9). The microsporidian spore wall and the fungal cell wall, viewed by electron microscopy, appear to be transparent when post-fixed by the conventional osmium tetroxide method. Since post-fixation with RuO_4 reveals new microsporidian spore wall details and establishes distinct ultrastructural differences among microsporidian species (7,8), it possibly also will reveal new ultrastructural features of the fungal cell. The present study was designed to evaluate the comparative effect of OsO_4 and RuO_4 post-fixing on fungal ultrastructure, particularly on their cell walls.

The fungal cell wall is a very important constituent and protective structure (10,11). The wall, more than any other cellular organelle, taxonomically defines fungi and distinguishes them from other organisms. Knowledge of fungal cell wall structure is of interest for taxonomy (12) and in the search for potential target sites for new chemotherapeutical agents (13).

Materials and methods

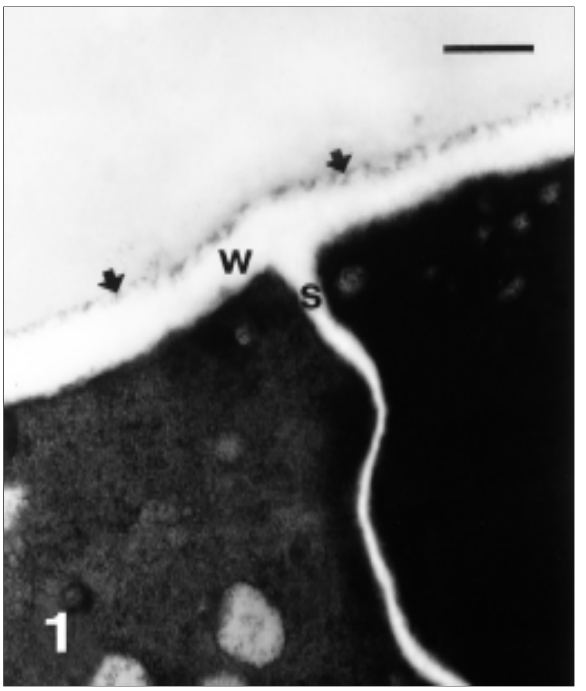
Saccharomyces cerevisiae yeasts, maintained in distilled water at 4 ºC for several weeks, and *Penicillium* sp. mycelia, growing on decaying tangerine, were used for this study. For the electron microscopy of yeasts, a yeast pellet was obtained by centrifugation and processed by dehydration and embedding, with centrifugation after each step. Mycelial mats were processed including the whole plant substrate. All fixatives were buffered in 0.13 M phosphate buffer (14). The same buffer was used for rinsing after each fixing step. The samples were first fixed for 24 hours in 3% glutaraldehyde, then washed for 12 hours. Three groups of samples from each fungal species were post-fixed separately in the following solutions: 1) 1% OsO_4, 1 hour; 2) 0.2% RuO_4, 1 hour; 3) 1% OsO_4, 1 hour followed by 0.2% RuO_4, 1 hour. After OsO_4 or RuO_4 post-fixing, the samples were washed several times for 2 hours. They were then dehydrated in a graded ethanol series (50, 70, 96 and 100%) 20 min/change, and infiltrated with resin:solvent (Epon-Spurr: propylene oxide) mixtures (1:2; 1:1; 2:1) at 12 hour intervals until embedding in pure resin Epon-Spurr mixture (15). Embedded capsules were then polymerized at 68 °C for 48 hours. Thin sections were stained with uranyl acetate and lead citrate and then examined with a Zeiss EM 109® electron microscope. Representative thin sections were observed unstained.

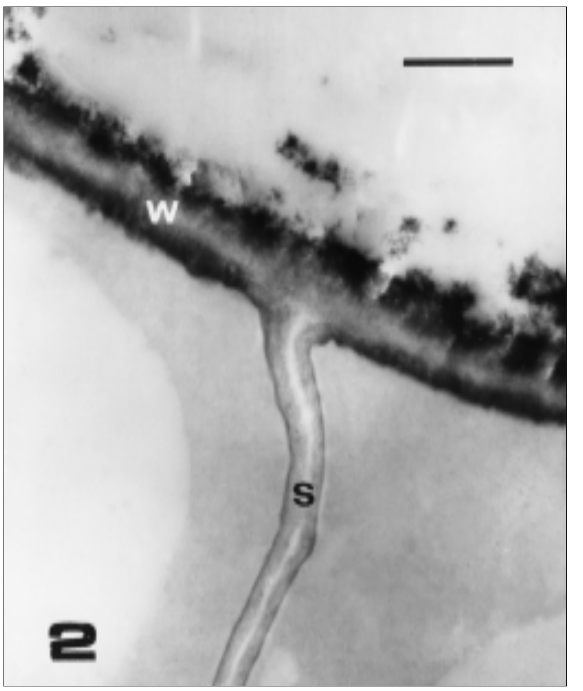
Results

Both hyphae and yeasts exhibited a different appearance after OsO_4 or RuO_4 post-fixing. The fungal OsO_4 post-fixed cell wall showed an electron-lucent thick band sometimes bordered by a thin electron-dense layer (figures 1, 3, 5, 7). RuO_4 or OsO_4 followed by RuO_4 changed the fungal cell wall's ultrastructural image, both on hyphae (figures 2, 6) and yeasts (figures 4, 8-12). RuO_4 post-fixing revealed fungal cell walls with two or three graded electron-dense layers (figures 2, 4, 6). Two other prominent features were observed. First, septa *of Penicillium* hyphae appeared as three layers composed of two grey layers separated by a translucent layer. The grey layers were seen as an invagination of the cell wall's inner layer (figure 2). The second feature concerned filiform protrusions emerging from the inner layer of budding *S. cerevisiae* yeasts' cell walls, and intruding into the cytoplasm (figures 4, 12). These protrusions seemed to originate from plasma membrane invaginations (figures 9-11) which were then penetrated by cell wall material. The cell wall showed a more homogeneous appearance in the double post-fixed samples (OsO_4 plus RuO_4), indicating that the internal fungal cell ultrastructure was better preserved. In budding yeasts, however, the cell wall protrusions were not always distinguishable from the cytoplasm (figure 8).

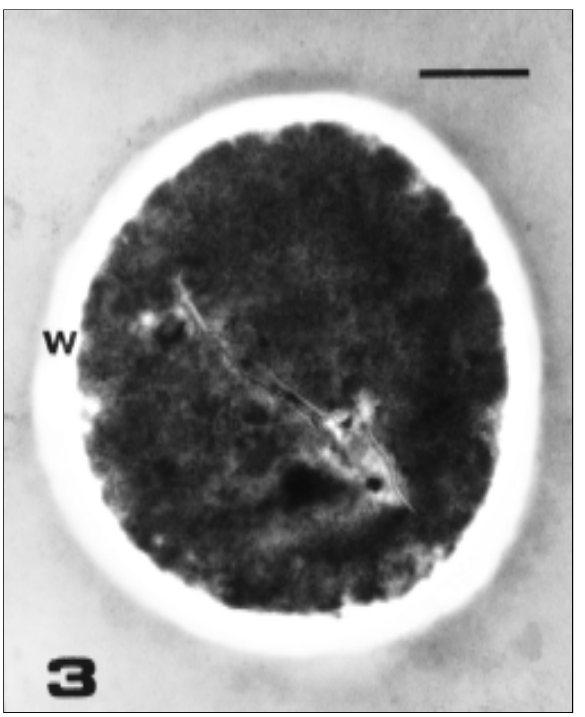
Discussion

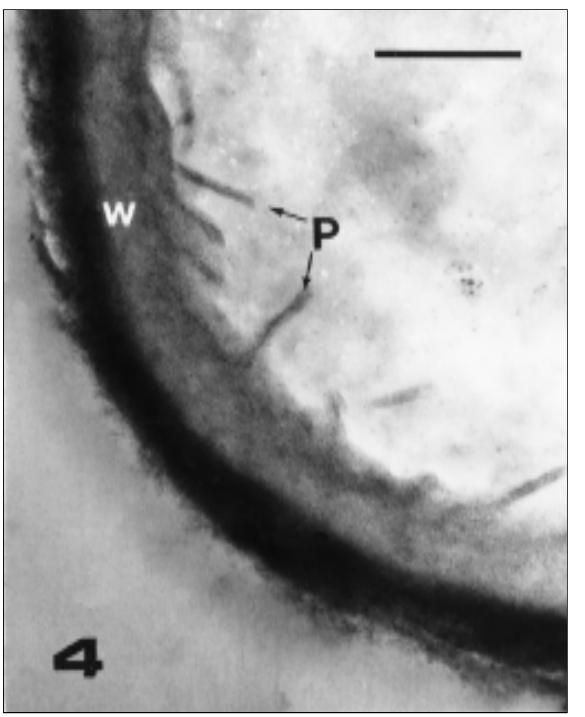
The apparent electron transparencies on the fungal cell wall may be due to deficient post-fixing by the conventional OsO_4 method. The polysaccharides chitin and glucan are the main fungal cell wall components (10,11). Because RuO_4 is a stronger oxidizing agent than OsO_4 (3,4), it probably reacts better with polysaccharide hydroxyl groups (7). The fungal cell wall also contains saturated fatty acids that perhaps confer hydrophobicity (10,12). RuO_4 post-fixing revealed saturated lip-



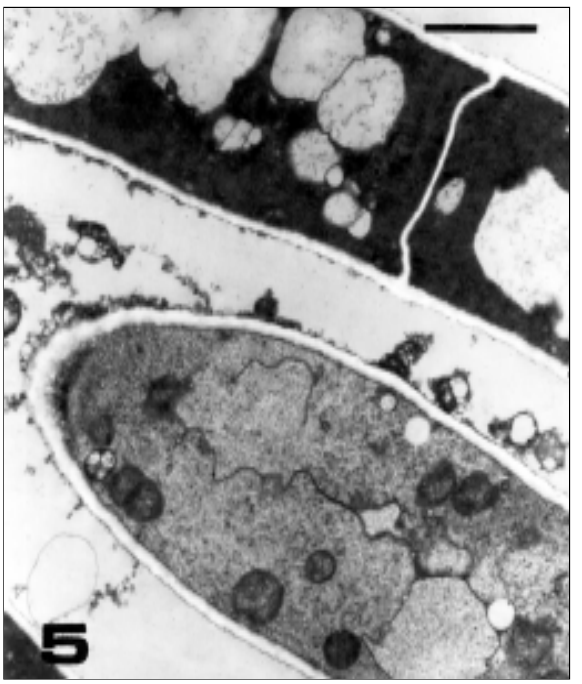


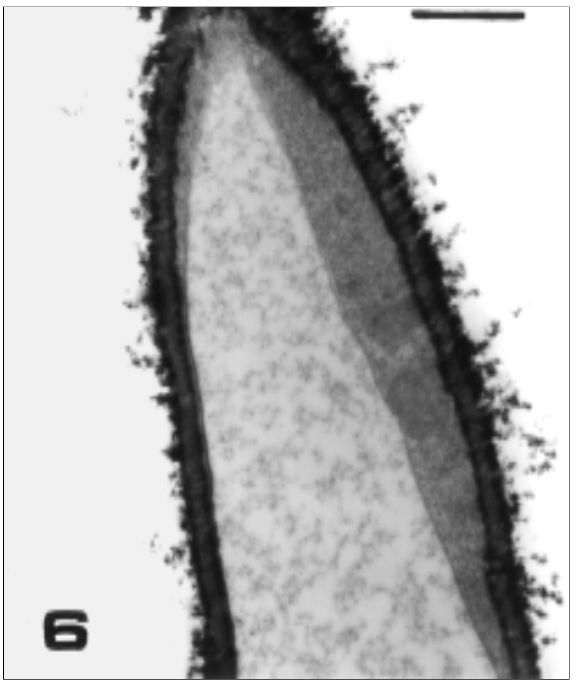
Figures 1 and 2. Cell walls and septa of *Penicillium* sp. hyphae. **Figure 1.** OsO_4 post-fixed specimen: the cell wall and the septum are transparent. Note the border (arrows). Scale bar, 0.4 µm. **Figure 2.** RuO_4 post-fixed specimen: the cell wall and the septum show a three-layered image. W: cell wall; S: septum. Scale bar, 0.3 µm.



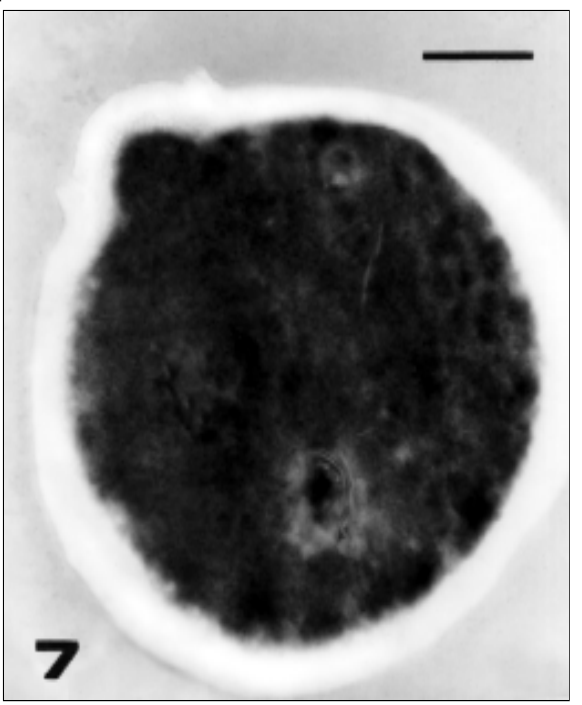


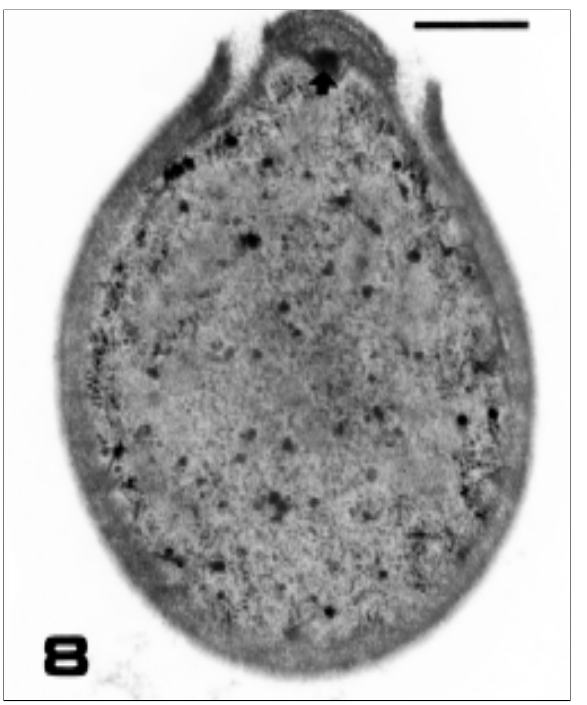
Figures 3 and 4. *Saccharomyces cerevisiae* yeasts. **Figure 3.** OsO_4 post-fixed yeast: the cell wall is translucent**.** Scale bar, 0.5 µm. **Figure 4.** Part of a budding yeast post-fixed with RuO_4: the cell wall is electron-dense. Note the filiform protrusions (P) intruding into the cytoplasm. W: cell wall. Scale bar, 0.2 µm.



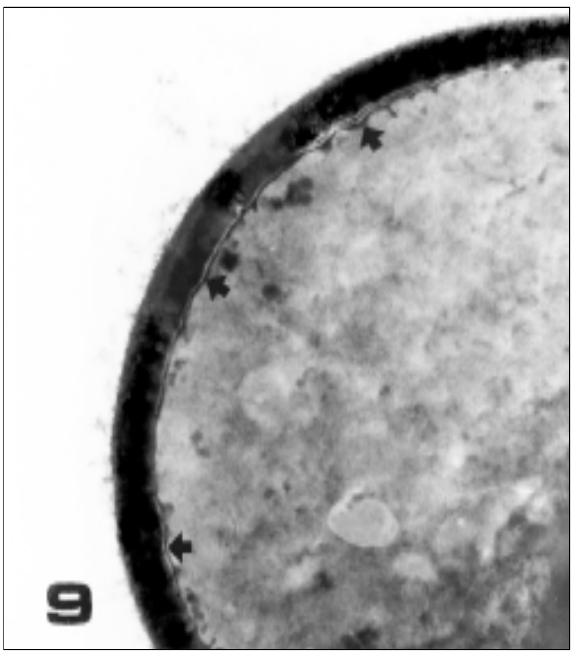


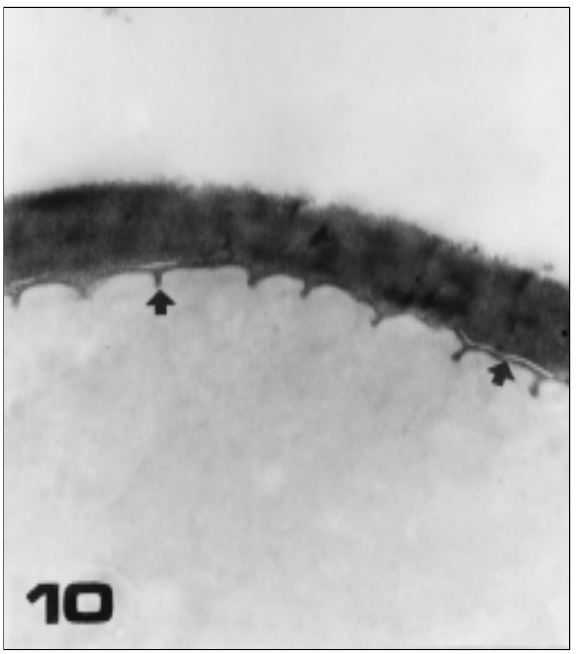
Figures 5 and 6. *Penicillium* sp. hyphae. **Figure 5.** OsO_4 post-fixed hyphae showing electron-transparency of the cell wall and septum. Scale bar, 1.5 µm. **Figure 6.** RuO_4 post-fixed specimen showing electron-dense cell wall. Scale bar, 0.8 µm.



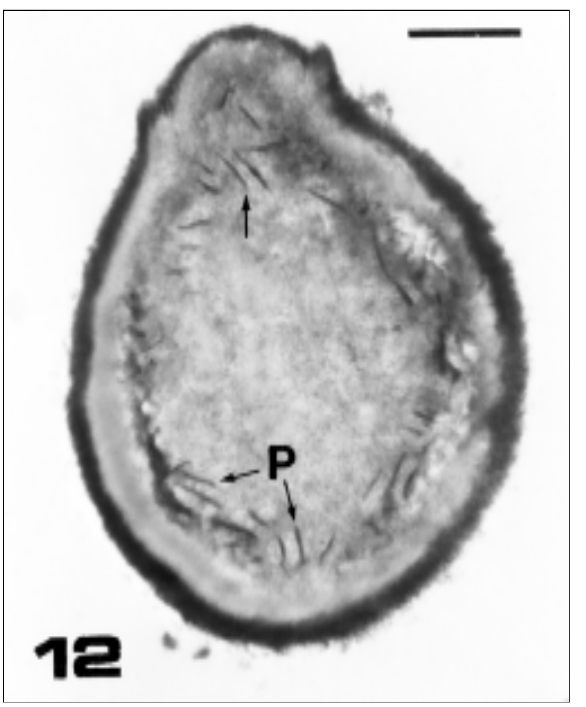


Figures 7 and 8. Budding yeasts of *S. cerevisiae*. **Figure 7.** OsO_4 post-fixed yeast displaying electron-lucent cell wall. Scale bar, 0.5 µm. **Figure 8.** Yeast post-fixed by OsO_4 followed by RuO_4 displaying electron-opaque cell wall. Note the Woronin body (arrow). Scale bar, 0.6 µm.





Figures 9 and 10. Cell wall of a *S. cerevisiae* yeast post-fixed with RuO_4. Note the beginning of plasma membrane invaginations (arrows). **Figure 9.** Scale bar, 0.3 µm. **Figure 10.** Scale bar, 0.2 µm.



Figures 11 and 12. Budding yeasts of *S. cerevisiae* post-fixed with RuO_4. **Figure 11.** The filiform protrusions (arrows) emerge from the cell wall. Scale bar, 0.6 µm. **Figure 12.** The filiform protrusions (P) emerge from a thicker cell wall. Scale bar, 0.5 µm.

ids which OsO_4 did not (5,6). The electron opacity observed on the fungal cell wall following post-fixing with RuO_4 therefore may be explained by the ability of this fixative to react with polysaccharides and saturated lipids. Differences in the electron density in each of the cell wall layers suggested different polysaccharide and lipid distributions.

Other electron microscopy methods have previously been used for fungal cell wall staining. *Candida albicans* yeasts have shown a two-layered electron-dense cell wall in samples fixed with glutaraldehyde-osmium tetroxide and stained using the periodic acid-thiocarbo-hydrazide-Ag-proteinate (PATAg) technique (16). PATAg is a specific cytochemical method for revealing polysaccharides by electron microscopy (17). In other studies, $KMnO_4$ fixing has produced light electron-density staining in the wall of young cells from some fungal species (18,19). Recently, *Ustilago maydis* hyphae, fixed with $KMnO_4$ followed by OsO_4, were shown to have cell walls with several distinct layers with differing electron densities. This treatment stained only the outer surface layer of yeast cell walls, which is not the case with mycelial walls from the same species (20). Several authors have obtained sharp ultrastructural yeast images by conventional methods but these images do not show adequate contrast in the cell wall (21-23). On the other hand, the yeast cell wall post-fixed with OsO4 was stained after enzyme treatment (24,25); however, this procedure requires removing carbohydrates from the cell wall and proteins within the cell (25).

High fungal cell wall contrast has been obtained with the method described here. The results agree with previous descriptions of the microsporidian spore wall (7,8). RuO_4 penetration of the mature cell wall revealed three-layered *Penicillium* septa. This method also enabled visualization of the filiform cell wall protrusions that extend into the cytoplasm in budding *S. cerevisiae.* Previously, these ultrastructural features had been unnoticed. The filiform cell wall protrusions seem to originate in the plasma membrane invaginations which are then penetrated by the cell wall material. Other authors have reported that RuO_4 reacts with superficial polysaccharide fibrils from reverting yeast protoplasts without penetrating intact cells (4,26). A recent review on preparation of yeasts cells for transmission electron microscopy includes OsO_4 and $KMnO_4$ but not RuO_4 post-fixing (25). Our results demonstrated RuO_4 reactivity with intact and mature fungal cell walls. Post-fixing procedures, including RuO_4 combined with OsO_4, are therefore recommended for ultrastructural studies of clinically important fungi.

References

1. **Sabatini DD, Bensch K, Barnett RJ.** Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J Cell Biol 1963;17:19-58.
2. **Gaylarde P, Sarkany I.** Ruthenium tetroxide for fixing and staining cytoplasmic membranes. Science 1968; 161:1157-8.
3. **Hayat MA.** Fixation for electron microscopy. New York: Academic Press Inc; 1981. p.194-5.
4. **Robards AW, Wilson AJ.** Procedures in electron microscopy. Volume 1. York, U.K: John Wiley & Sons; 1993. p.36-38, 54-59.
5. **Madison KC, Swartzendruber DC, Wertz PW, Downing DT.** Presence of intact intercellular lipid lamellae in the upper layers of the stratum corneum. J Invest Dermatol 1987;88:714-8.
6. **Van den Bergh BAI, Swartzendruber DC, Bos-Van der Geest A, Hoogstraate JJ, Schrijvers AHG, Boddé HE, *et al.*** Development of an optimal protocol for the ultrastructural examination of skin by transmission electron microscopy. J Microsc 1997;187: 125-33.
7. **Torres-Fernández O.** Ultrastructural findings on microsporidia spore wall as seen by ruthenium tetroxide fixation. J Invertebr Pathol 2001;77:138-40.
8. **Torres-Fernández O.** Microsporidian spore: new electron microscope appearance. Biomédica 2001;21: 310-2.
9. **Weiss L, Edlind T, Vossbrinck C, Hashimoto T.** Microsporidian molecular phylogeny: the fungal connection. J Eukaryot Microbiol 1999;46:17S.
10. **Ruiz-Herrera J, Sentandreu R.** Fungal cell wall synthesis and assembly. En: McGinnis MR, Borges M, editors. Advances in Medical Mycology. Vol. 13. New York: Springer-Verlag; 1989. p.168-217.
11. **Fontaine T, Mouyna I, Hartland RP, Paris S, Latgé JP.** From the surface to the inner layer of the fungal cell wall. Biochem Soc Trans 1997;25:194-9.
12. **Bartnicki-García S.** Cell wall chemistry, morpho-genesis, and taxonomy of fungi. Ann Rev Microbiol 1968; 22:87-108.

13. **Gozalbo D, Elorza MV, Sanjuan R, Marcilla A, Valentin E, Sentandreu R.** Critical steps in fungal cell wall synthesis: strategies for their inhibition. Pharmacol Ther 1993;60:337-45.

14. **Milloning G.** Advantages of a phosphate buffer for OsO_4 solutions in fixation. J Appl Phys 1961;32:1637-9.

15. **Hazard EI, Ellis E, Joslyn D.** Identification of Microsporidia. En: Burges HD, editor. Microbial control of pests and plant diseases, 1970-1980. New York: Academic Press; 1981. p.163-82.

16. **Moulin-Traffort J, Venot C, Regli P.** Action del l'soconazole sur *Candida albicans.* Étude en microscopie électronique á balayage et á transmission. Path Biol 1986;34:899-907.

17. **Thiéry JP.** Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. J Micr (Paris) 1967;6:978-1018.

18. **Illingworth RF, Rose AH, Beckett A.** Changes in the lipid composition and fine structure of *Saccharomyces cerevisiae* during ascus formation. J Bacteriol 1973;113: 373-86.

19. **Kreger-Van Rij NJW, Veenhuis M.** Electron microscopy of some special cell contact in yeasts. J Bacteriol 1973;113:350-6.

20. **Ruiz-Herrera J, León CG, Carabez-Trejo A, Reyes-Salinas E.** Structure and chemical composition of the cell walls from the haploid yeast and mycelial forms of *Ustilago maydis*. Fungal Genet Biol 1996;20:133-42.

21. **Sentandreu R, Northcote DH.** The formation of buds in yeast. J Gen Microbiol 1969;55:393-8.

22. **Slater M, Bowers B, Cabib, E.** Formation of septum-like structures at locations remote from the budding sites in cytokinesis-defective mutants of *Saccharomyces cerevisiae*. J Bacteriol 1985;162:763-7.

23. **Tuinen E, Riezman H.** Immunolocalization of glyceraldehyde-3-phosphate dehydrogenase, hexokinase, and carboxypeptidase Y in yeast cells at the ultrastructural level. J Histochem Cytochem 1987;327-33.

24. **Zlotnik H, Fernández MP, Bowers B, Cabib E.** *Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determines wall porosity. J Bacteriol 1984;159:1018-26.

25. **Wright R.** Transmission electron microscopy of yeast. Microsc Res Tech 2000;51:496-510.

26. **Osumi M.** The ultrastructure of yeasts: cell wall structure and formation. Micron 1998;29:207-33.