The Development of the Electron Microscope and Its Application to the Brewing Industry[1]

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ABSTRACT

The electron microscope has been a multipurpose tool for many of the basic sciences, but its practical application in industry is only now being considered. This review article examines the basic techniques employed in electron microscopy and the basic functioning of the instrument with respect to recent multidetector analyses. Past, present, and future uses of the instrument in the brewing industry are discussed.

Key words: Analytical review, Genetics, Microbiology

The science of electron microscopy has grown rapidly since Seimens introduced the commercial electron microscope (EM) in 1939 (26). Scientists from diverse disciplines have benefited from the ability of the EM to extend the observable range of structure beyond the limits of visible and ultraviolet light. The shorter wavelength of electrons permits the resolution of objects as small as 0.2 nm or smaller, whereas light microscopes have a resolving limit of only 2,000 nm, depending on the wavelength of light used. When used with biological specimens, the resolving power of most modern transmission electron microscopes (TEMs) is from 1.0 to 2.5 nm, due to a number of factors (eg, biological specimens and their support films tend to increase electron scattering, and this results in decreased resolutions). In addition to the increased resolving power of the EM, the variety of signals produced by electron beam and specimen interactions (ie, X-rays and inelastically scattered electrons) can provide useful information about the composition of the specimen.

HISTORY OF THE EM IN BREWING

Two years after the EM was introduced, Rhea (42) discussed the operation of the TEM and its possible applications in brewing research. Around the same time, Mudd et al (27) employed the TEM to perform crude morphological studies on several bacteria, including an occasional beer contaminant, *Pediococcus cerevisiae*. The electron micrographs in pioneering studies of this kind provided little information other than the general morphology of the specimens as a result of unrefined preparatory techniques.

In 1943, Fairs (12) used the TEM to determine particle sizes of powders such as diatomaceous earth. Correlations were made between particle size and shape and the effect of these two factors on filterability. A similar study by Clarke and Lawrie (8) used the TEM to study shape and sizes of colloidal beer proteins and compounds such as ferric chloride and tannic acid.

From 1944 to 1949, TEM technology advanced dramatically (26). During the same period, however, activity with the TEM in brewing ceased. Upon the development of more refined technologies, TEM research in brewing was renewed with an investigation of beer particles, particularly crystalline yeast vitamins (2). Van Itterbeek and co-workers (52) continued work begun earlier on colloidal beer proteins, and their research led to the correlation of beer colloidal materials with beer hazes.

Refinements in the instrument and in preparatory techniques enabled brewing researchers to investigate yeast cell ultrastructure in greater detail. In 1953, two separate groups began research on brewing yeast cell ultrastructure. Houwink and Kreger (17) examined fine structure of the yeast cell wall, while Bartholomew and Mittwer (3) examined yeast bud scars. A short time later, Bartholomew and Mittwer (4) extended their original research with an examination of nucleosomal changes during bud formation. In 1959 Agar and Douglas (1) provided the first electron micrographs produced by TEM that clearly resolved internal cytoplasmic structures (ie, nuclear membranes and pores). Their report contains a series of electron micrographs illustrating the complete cycle of budding in *Saccharomyces cerevisiae* and the corresponding cytoplasmic changes.

In 1963, an expansion of studies with the TEM took place in the brewing industry. Claussen and Sandegren (9) demonstrated a relationship between chill hazes and particle shape. Chill haze particles were found to be spherical and to have similar sizes, whereas permanent haze particles were irregular in both shape and size. Research in yeast ultrastructure was continued by Rose (44), who correlated yeast biochemistry with ultrastructure. In the same year, a new approach in brewing research was initiated by Buttrose (6), who investigated the fine structure of brewing materials. Aleurone cells of germinated barley and wheat were examined structurally by TEM to determine optimal conditions for germination. Research on barley aleurone cells was continued in several laboratories through the 1960s (7,11,18,19,25,31,35,50).

The first use of the scanning electron microscope (SEM) in brewing research appeared in 1972 in a preliminary report (32) from a series of studies by Palmer (33–35). Both the transmission and the scanning electron micrographs Palmer presented showed the degradation of starch gradules during mashing. In these studies, a fourth dimension was added to the normal three-dimensional effect of scanning electron micrographs. The added dimension was time, ie, the effect of mashing time on starch degradation.

Numerous studies using the SEM followed these first investigations. Studies of surface morphology of barley (37,47), oats (39), and wheat (38) during germination were conducted in several brewing laboratories. Palmer used the SEM to study the effect of tumbling and abrasion on enzyme formation in malting grain (33,34). The SEM has also been used in bacterial taxonomy for discerning the flagellar arrangement of *Pectinatus*, a new bacterial isolate (22).

The TEM continued to be used for brewing research by such workers as Williams and Duffus (56), who investigated the development of endosperm during barley maturation. A communication by Von Wettstein (53) in 1979 heralded an entirely new use for the TEM in brewing research. This research dealt with the application of new genetic techniques to the manipulation of malting barley and brewing yeasts. The equipment for monitoring genetic manipulation of yeasts included the TEM. As bioengineering and biotechnology have become more important in brewing, the TEM and the new-generation scanning transmission electron microscopes (STEMs) have become valuable tools in correlating ultrastructural changes with genetic transformations (21).

INSTRUMENTATION

The three types of electron microscopes—transmission, scanning, and scanning transmission—are categorized according to their mode of operation. The three types are briefly described here to outline differences in their modes of operation and applications, but an extensive discussion of the electronics, vacuum systems, and electron optics of the various instruments is beyond the scope of this review. Meek's comprehensive book on electron microscopy (26) provides such information.

[1]Presented at the 49th Annual Meeting, Nashville, TN, April 1983.

Transmission Electron Microscopy

In a TEM, a series of magnetic and electrostatic lenses are stacked in a columnar arrangement (Fig. 1). The lenses, which can vary in number, and the photographic system are enclosed and maintained under high vacuum. Set on top of the column is the electron source, usually a tungsten filament enclosed in a shield. The shield, called a Wehnelt, is held at a negative potential relative to the filament. This creates an electron cloud around the circular opening at the center of the Wehnelt. The electrons from this region are then accelerated by the anode down through the column and specimen. Initially the condenser lens focuses the electron beam, and this provides the intensity of illumination required to view the specimen, which is placed in or just above the objective lens. Although the specimen will absorb some of the electrons, many pass through it and enter the objective lens where a magnified image is produced. The projector lens, in conjunction with the diffraction and intermediate lenses, provides further magnification of the image, which can then be projected on a fluorescent screen or recorded on a photographic emulsion.

Resolution of approximately 0.3–0.4 nm (point to point) can be achieved with the TEMs currently available. Unfortunately, this kind of resolution is not yet obtainable with biological specimens, where the resolution is generally one to two orders of magnitude greater. The TEM is, however, an excellent instrument for routine structural observation. A typical transmission electron micrograph from a thin section of the gram-positive bacterium *Pediococcus damnosus* var *II* is given in Fig. 2.

Perhaps the greatest limitation of TEMs is the fact that they depend on the passage or transmission of electrons through a specimen. For this to occur, the specimen must exist either as a thin section or a thin film that, by necessity, has been killed in the process of preservation. SEMs, on the other hand, can allow examination of entire specimens and, in some cases, specimens that have not been subjected to killing or preservation (14,40).

Scanning Electron Microscopy

Image formation in the SEM is discussed in detail by Newbury (29) and Meek (26). In image formation, a fine beam of electrons is focused on the surface of a specimen and scanned over an area in a zigzag pattern similar to that on a television screen raster. The secondary or backscattered electrons that leave the surface of the specimen are then collected by an appropriately placed detector. The current produced is then amplified and a signal displayed on a cathode ray tube (CRT, Fig. 3). Because the signals displayed on the CRT are synchronous with the position of the electron beams on the surface of the specimen, the picture obtained appears to be magnified and three-dimensional. A scanning electron micrograph of *S. cerevisiae* (Fig. 4) shows the three-dimensional appearance of

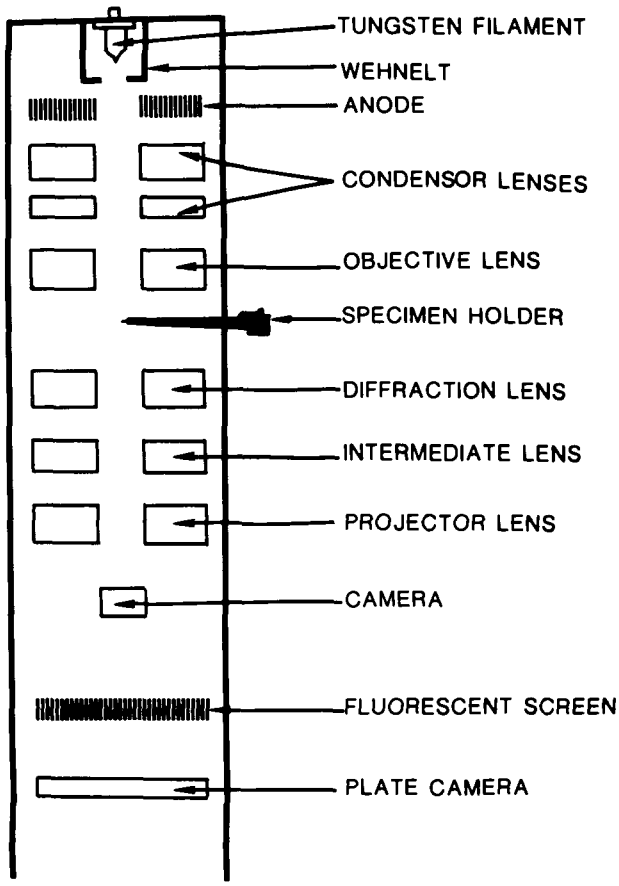


Fig. 1. Schematic diagram of a transmission electron microscope.

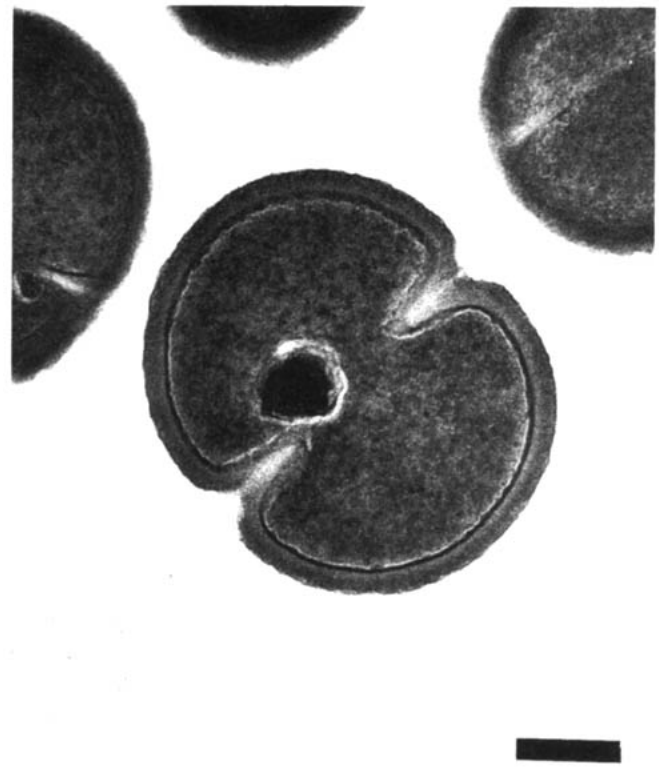


Fig. 2. Transmission electron micrograph of *Pediococcus damnosus* var *II* (fixed in 5% glutaraldehyde and 1% OsO_4). Bar = 100 nm.

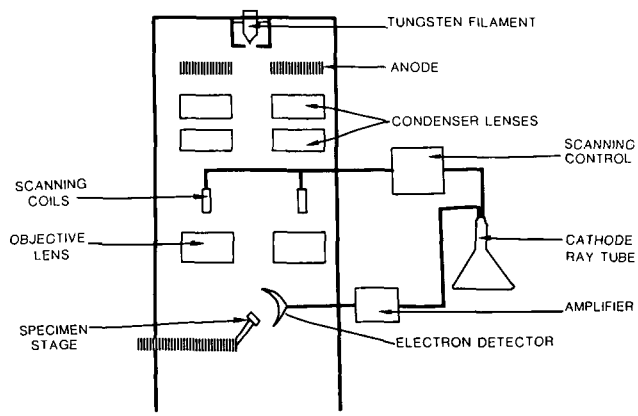


Fig. 3. Schematic diagram of a scanning electron microscope.

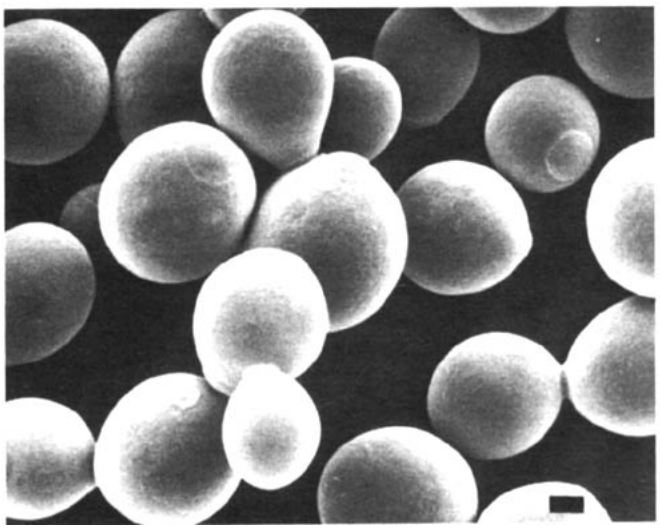
the image.

Although the resolution obtained with the SEM (8–9 nm) does not compare with that obtained with the TEM (0.3–0.4 nm), magnifications up to 60,000× are obtainable. A scanning electron micrograph of a crystal (Fig. 5) illustrates the most valuable feature of the SEM, its great depth of focus.

Scanning Transmission Electron Microscopy

The STEM is the most sophisticated microscope available. In a broad sense, an STEM is a TEM equipped with attachments that allow it to function in a mode similar to that of the SEM (26,54). A sharply focused electron beam is scanned across a thin specimen of the type used in a TEM. The emissions generated by electron beam-specimen interactions are captured by a variety of detectors placed beneath or around the specimen (Fig. 6). The signals generated can be processed and displayed in a variety of ways, including bright-field, dark-field, and X-ray spectra. The STEM has no need of imaging lenses and can obtain magnifications in the order of millions without image distortion. Resolving power is simply determined by the diameter of the electron beam spot.

STEMs have several advantages, one of which is dark-field capabilities that produce images relatively free of interference from noise. Another is fine control of the irradiation dose to which the surface of the specimen is exposed (ie, minimum electron beam damage). However, the major advantage of STEMs is their ability



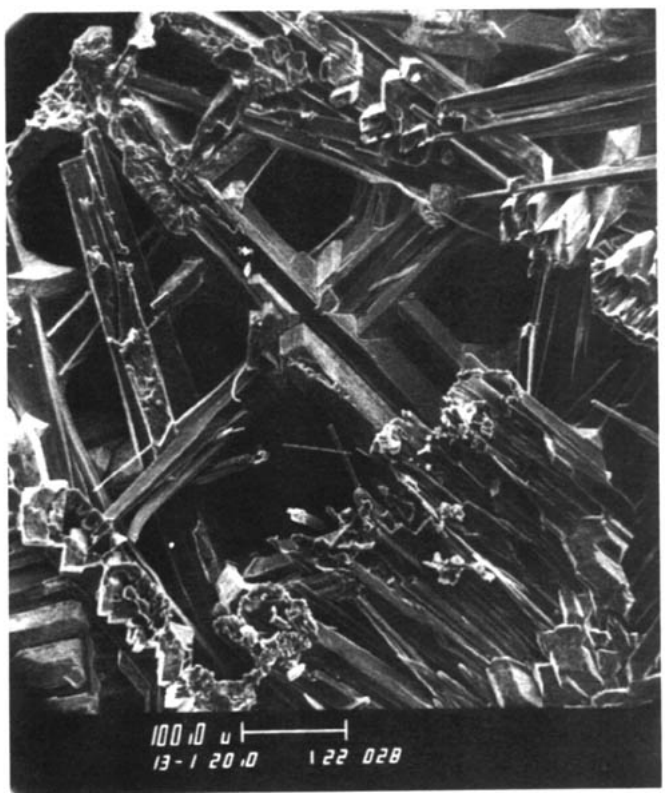


Fig. 5. Scanning electron micrograph of a crystal of the mordant potassium chlorate. (Photograph courtesy of L. Ackland)

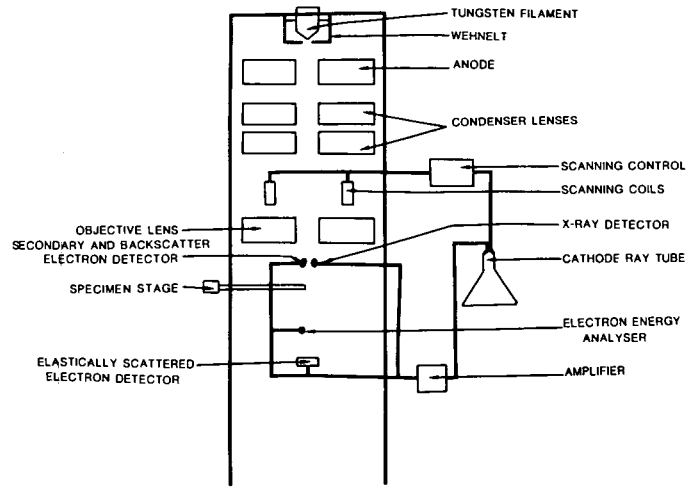


Fig. 6. Schematic diagram of scanning transmission electron microscope equipped with a multidetector.

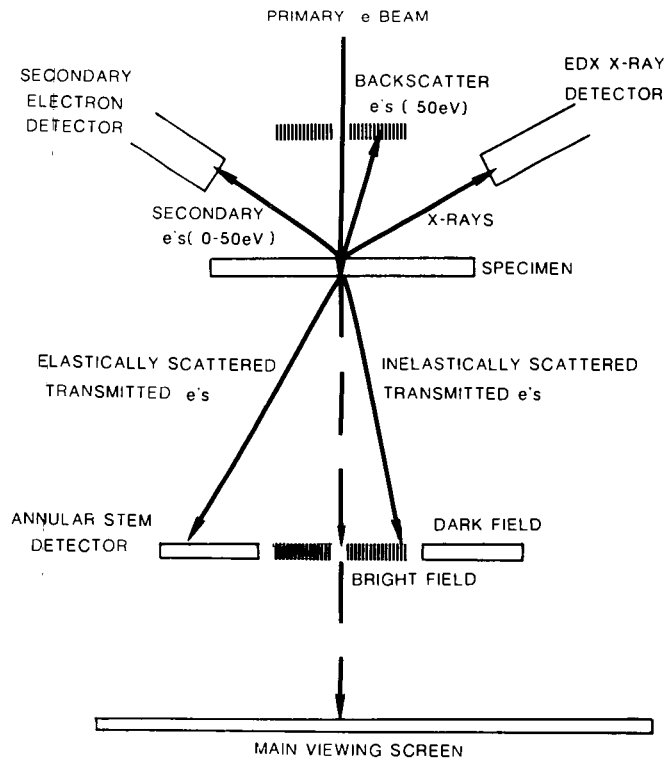


Fig. 7. Signals produced and subsequently analyzed in a scanning transmission electron microscope.

to perform elemental microanalysis by energy-dispersive X-ray spectroscopy (EDX) and by electron energy loss spectroscopy (EELS, 30,45,51). Figure 7 illustrates the variety of signals the STEM uses for many analytical techniques.

The STEM is not simply a combined TEM-SEM high-resolution system; it is also an analytical device capable of deriving compositional information from a specimen. The great advantage of the STEM to biologists is its ability to analyze a specimen image into separate channels, all of which contain valuable information that can be processed and displayed separately.

PREPARATION OF BIOLOGICAL SPECIMENS

Fixation

A biological specimen usually cannot be placed directly into the electron beam of the EM without prior preparation. The first step in preparing a specimen for the TEM is to fix the specimen, to prevent structural alterations and distortion from subsequent exposures to organic solvents and embedding plastics. The fixative of choice is usually glutaraldehyde in low concentrations or glutaraldehyde vapors in a closed vessel. Osmium tetroxide (OsO_4) is another commonly used fixative (and stain) for electron microscopy.

The next step is dehydration, a procedure whereby the specimen is subjected to increasing concentrations of ethanol from 30% v/v to absolute. As a result, the water in the sample is replaced with ethanol. After this occurs, the specimen may be embedded in a water-immiscible plastic. This process makes the specimen more amenable to thin sectioning by supporting the specimen in a block of rigid plastic material. An unpolymerized plastic compound is added to the dehydrated specimen and allowed to completely permeate the specimen. After a suitable period of incubation, the mixture can be cured in an oven.

The final stage of preparation is thin sectioning of the specimen. The specimen must be sectioned as in light microscopy, but unlike light microscopy, the sections must be very thin—less than 0.1 mm. The embedded specimen provides the electron microscopist with a hard, rigid material that can be cut on a special instrument (an ultramicrotome) equipped with a glass or diamond cutting knife. Once cut, the sections are collected on a copper mesh grid (2.3–to 3.0 mm in diameter) that can then be placed into the TEM.

In the exacting science of microscopy, one must always be concerned with the production of artifacts. Fixation, dehydration, and subsequent staining may cause structural aberrations or deformations. Investigators have therefore exhaustively sought alternate methods for preparing specimens for imaging in the TEM.

One alternative to chemical fixation is cryogenic preservation (13,36,46). The specimen is "fixed" by quick freezing followed by sublimation at subzero temperatures of the surrounding aqueous phase or by dissolving the ice formed on freezing by substitution with a more suitable material at subzero temperatures. The specimen is then embedded in plastic before thin sectioning. The embedding may, however, cause deformations in the sample, as in chemical fixation.

A method developed by Virdon (26) utilizes freeze fixation of specimens without plastic embedding, thereby eliminating structural damage caused by embedding. This technique, called cryo-ultramicrotomy, involves thin sectioning of frozen samples at temperatures below −80°C. With the use of a cryotransfer unit, a frozen thin section can be placed in the EM without melting or thawing the sample before imaging. Although this technique has primarily been used to study mammalian tissues, recent investigations have employed cryo-ultramicrotomy to localize β-galactosidase and alkaline phosphatase in *Escherichia coli* (24).

Sample preparation for the SEM is less complex than that for the TEM. The specimen does not have to be thin sectioned before imaging because SEM signals are generated at the surface of the specimens. Usually, the specimen is dehydrated with ethanol solutions of increasing concentrations, as in the TEM; however, no prefixing or staining is required. The specimen is then critical point-dried. In this procedure, the ethanol, which has replaced the water of the specimen, is displaced by CO_2 under high pressure and varying temperatures. Subsequently, the specimen is coated under vacuum with a thin film of gold palladium, which renders the surface electron reflective. The specimen can then be placed in the SEM for imaging.

Staining

To provide electron contrast, a specimen is often stained with an electron-dense substance. The heavy metal that reacts with the specimen produces a "shadow gram" in the EM. The metal deflects or prevents passage of electrons through the specimen and results in a corresponding dark spot in the final micrograph. The most commonly used materials are the salts of heavy metals such as uranium and lead.

Two types of staining for biological material have been developed, positive and negative staining. Positive staining is the binding of an electron-scattering agent with reactive chemical sites in the specimen. The two most important stains in use are uranium or lead-based salts. OsO_4, which has been routinely used for many years as a stain for light microscopy (41,55), provides electron contrast (15) and is thought to act as a fixative for stabilizing lipid material. Specific stains are also available for monitoring cellular charge density or for staining specific biopolymers. Rutherium red, for instance, stains specific macropolysaccharides. Figure 8 illustrates positive staining.

Very little is known about the interactions between specimens and heavy metal salts. It is suspected that the isoelectric points of

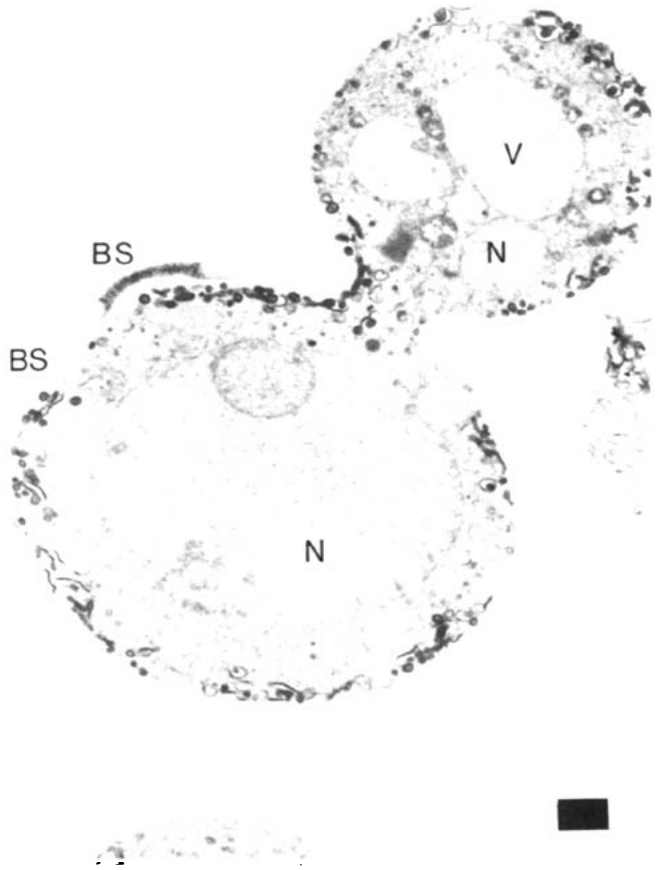


Fig. 8. Electron micrograph of *Saccharomyces cerevisiae* positively stained with uranyl acetate and osmium tetroxide. BS = birth scars, V = vacuole, N = nucleus. Bar = 400 nm. (Photograph courtesy of D. Von Wettstein and *EBC Proc.* for 1979, p. 614, Fig. 22)

some intracellular proteins fall well below those of the surrounding proteins to result in an anionic heavy metal sink (15). DNA and RNA are thought to react with heavy metal salts because of the high phosphate content of these compounds (15). Phosphate groups, which are also present at the hydrophilic surfaces of membranes, are anionically charged and could function as potent heavy metal chelators.

Negative staining uses electron-dense salts similar to those used for positive staining. The specimen is surrounded by a thin film of the electron-scattering agent by rapidly drying the heavy metal solution. The stain surrounding the specimen produces a "negative image" in the TEM. By definition, negative staining is an embedding of the biological material in a stain of high electron density (15). Negative staining is one of the most highly resolving techniques available for EM. Moreover, its simplicity allows negative stains to be used as a rapid method for examining cell morphology and for discerning the cell surface and its associated structures. A comparison of positive and negative staining of a similar specimen is presented in Figs. 9 and 10.

SPECIALIZED TECHNIQUES

A number of specialized techniques can be used in preparing or analyzing specimens in the EM. With these techniques, more information about morphology and biochemical composition can be discerned.

One specialized technique that uses freeze fixation of specimens is freeze fracturing (etching). Greater knowledge of three-dimensional structure can be obtained through this method. The specimen is frozen rapidly in liquid freon, held at liquid nitrogen temperatures, then fractured under vacuum. The fractured surface is shadowed with vaporized heavy metal and coated with a layer of carbon. The carbon replica is subsequently placed in an acid solution to remove residual cellular material and is then examined in the EM. Freeze etching does not rely on the use of chemical additives and, as a result, is thought to be a preparative technique capable of accurately preserving tissue in the "living" state (15). The technique is especially valuable because cells that are frozen remain viable and can be cultured upon thawing. Moreover, physical information can be derived from the images because cells cleave through areas of least bond energy, that is, along the hydrophobic centers of the membranes. Figures 11 and 12 illustrate freeze fracturing of the brewing yeast *S. cerevisiae*.

The freeze-etching technique has contributed much to our knowledge of prokaryotic and eukaryotic cell structure. Cell wall structure (49), mesosome structure (28), and the classification of bacterial spores (16) have all been reported through use of this method. Recent studies using this technique have been directed toward elucidation of three-dimensional structure in prokaryotic and eukaryotic cells (10).

Autoradiography is possible on the TEM and the STEM. A radioactive isotope label (^{3}H or ^{14}C) is incorporated into a cellular macromolecule, and the sample is prepared in the usual manner for electron microscopy. Before viewing, a fine-grain photographic emulsion is laid over the sample and incubated long enough that the emissions from the radioactively labeled areas expose the emulsion. The sample is photographically developed to reduce the exposed silver, which becomes fixed onto the specimen. Silver from the photographic emulsion, which pools over areas where the isotope is situated, can be imaged in the EM, allowing direct localization of the isotope in the specimen.

The use of autoradiography in the electron microscopy of prokaryotic cells is limited because of poor resolution, which arises from the large grain size of the photographic emulsions. Recent techniques developed for using this method on freeze-fracture specimens could improve the use of this method for examination of biological specimens (15).

Electron microscopic image enhancement is another recently

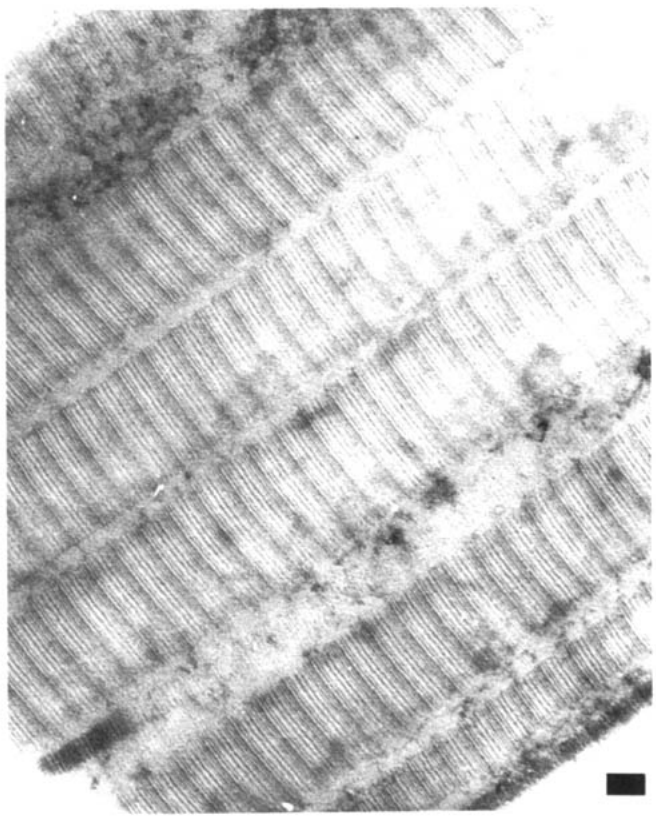


Fig. 9. Positively stained collagen fiber. Bar = 100 nm. (Photograph courtesy of P. R. Sweeney, University of Guelph)

Fig. 10. Negatively stained collagen fiber. Bar = 100 nm. (Photograph courtesy of P. R. Sweeney, University of Guelph)

developed technique. It is a method capable of eliminating much of the noise (ie, information that does not contribute to structural attributes) that prevents theoretical resolution from being attained in biological specimens. Through this method, the three-dimensional structure of the specimen can be discerned (Fig. 13). Unfortunately, at this time, this method can only be used for samples that are highly ordered (ie, symmetrical).

The EM can be used to localize specific enzymes of potential interest. One technique used to localize enzymes is very similar to the fluorescent antibody technique used in light microscopy. Instead of a fluorescent dye, an electron-opaque substance such as iron-containing ferritin (20) is conjugated with a specific antibody to the enzyme. This method is, of course, useful only when the enzyme or site of interest is on the cell surface.

A variation of this method has also been used to localize surface structures such as capsules and slimes. During hydration, these structures collapse and disappear. When preparations of antibodies against these structures are added to the specimen before fixing, the structures are stabilized and, with appropriate staining, can be viewed in the TEM.

STEMs are capable of performing a number of functions that turn the EM into an analytical tool able to discern chemical, physical, and morphological features. When the electron beam of an EM strikes a specimen, a number of energy-dispersion interactions may occur. These interactions can be used to provide useful chemical and physical information about the specimen.

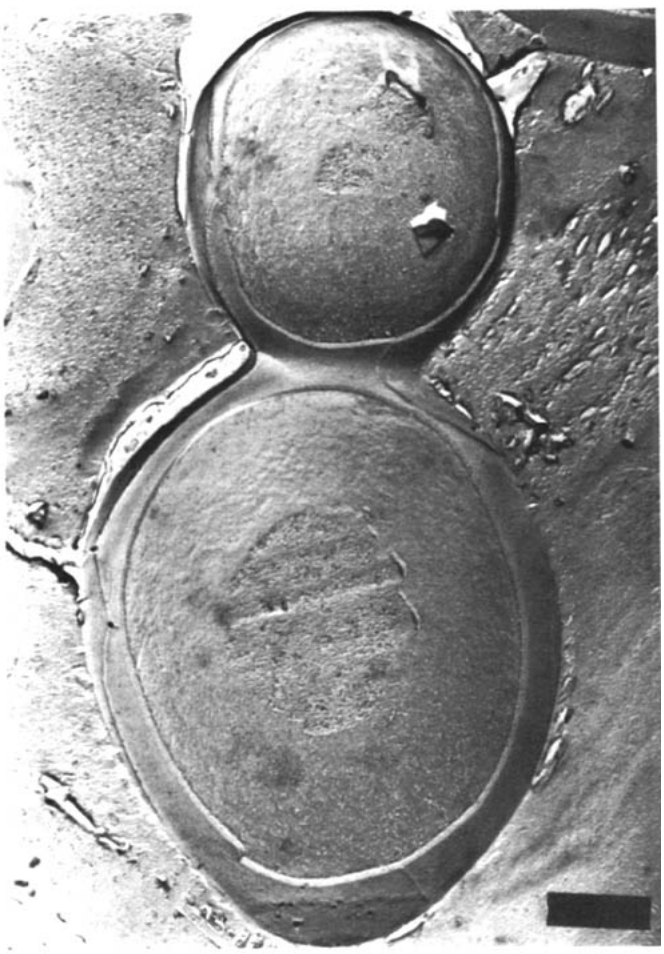


Fig. 11. Transmission electron micrograph of freeze-fractured *Saccharomyces cerevisiae*. Bar = 1,000 nm. Freeze fracturing has exposed the cell wall, the cell membrane, and a portion of the cytoplasm. (Photograph courtesy of D. Von Wettstein and *EBC Proc.* for 1979, p. 611, Fig. 19)

The EDX system for X-ray analysis is an example of an analytical system compatible with an STEM. X rays are released from a specimen because of electron rearrangements in the atomic energy shells that are generated by interactions between the electron beam and the specimen. Different energy levels of X rays are released, depending on what type of element reacts with the beam. Because of this, an X-ray spectrum is formed and can be measured by X-ray spectroscopy. Figure 14 shows an EDX spectrum of the electron micrograph of *P. damnosus* var *II* that was positively stained with uranyl acetate and OsO_4. The spectrum produced is a function of the atomic numbers of the elements in the specimen, the accelerating voltage of the instrument, and the beam current. This type of analysis is more suitable for the identification of elements with high atomic numbers than for the lighter elements (carbon, oxygen, nitrogen, and hydrogen), which are more abundant in biological tissues.

In X-ray analysis studies of biological material, a major problem is specimen preparation. Fixation and dehydration of specimens will extract or remove material from the sample. This reemphasizes the importance of cryo-ultramicrotomy. Figure 15 shows an X-ray analysis study of *P. damnosus* var *II*.

Another new elemental analysis systems, EELS, holds more promise for the study of biologically significant elements. EELS examines the energy distribution of electrons that have interacted with the specimen as they have passed through it. As with EDX, the elemental composition of the sample can be discerned. The electron energy-loss spectrum is collected by passing the dispersed electrons through a magnetic prism; electrons that have lost more energy (ie, those that have interacted more strongly with the specimen) are deflected more by the magnetic prism. This method is more

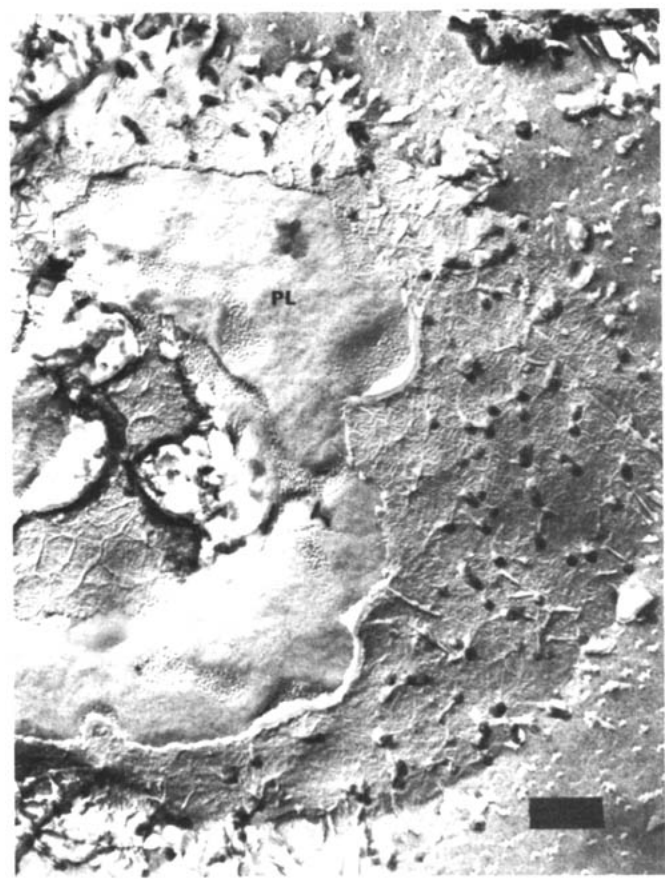


Fig. 12. Transmission electron micrograph of freeze-fractured *Saccharomyces cerevisiae* pretreated with an enzyme to partially digest the cell wall, exposing the plasmalemma (PL). Bar = 200 nm. (Photograph courtesy of D. Von Wettstein and *EBC Proc.* for 1979, p. 612, Fig. 20)

sensitive than EDX in that single atoms of compounds can be detected. In addition, the lighter elements, undetectable in EDX analysis, are detectable in EELS systems.

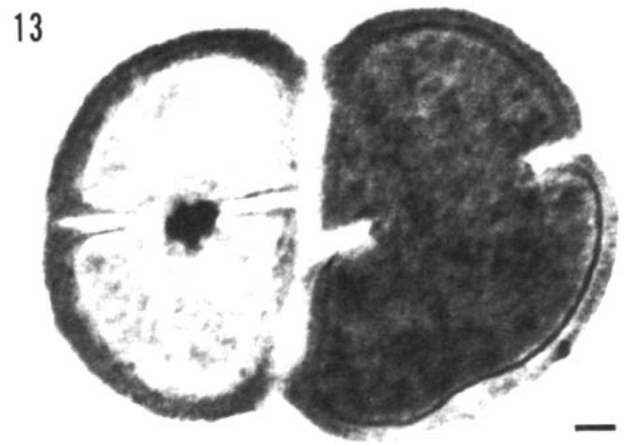
FUTURE OF THE EM

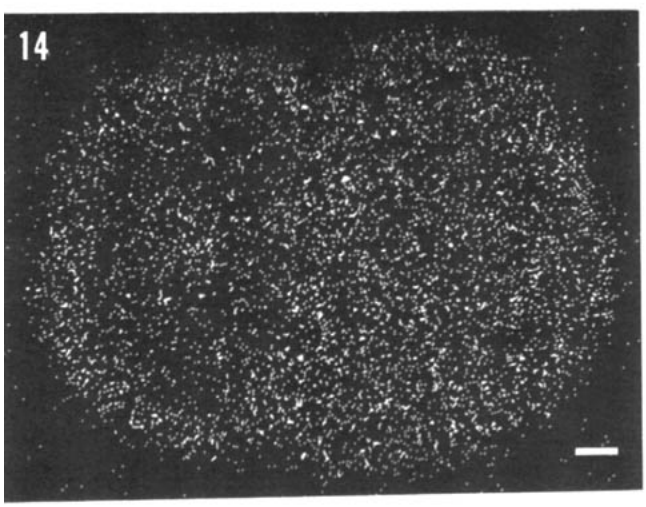
The EM undoubtedly will continue to be useful in examining the morphology of brewing materials. Through this kind of research, we may soon be able to develop superior malts by optimizing starch degradation, germination conditions, or malt handling. Continued use of the EM may help clarify or develop new concepts of hazes in beer.

The STEM, with its elemental analysis systems, will be an important tool in the future of brewing. Current brewing processes have been optimized as much as possible through equipment design and process control. The next logical step in improving brewing technology is to optimize the biological fermentation vector—the brewing yeast—through genetic manipulation. STEMs are valuable tools for determining biochemical and ultrastructural changes that, in turn, can be related to genetic manipulations (43,48). For example, structural gene products of important yeast genes (ie, *Flo* genes) may be localized by using data from integrated image and elemental analysis. Yeast cell composition can be further elucidated by using some of the new equipment available. A better understanding of the compartmentalization of yeast metabolism could result from studies of this kind.

The STEM could also be used to analyze for and to identify foreign matter in beer, in a similar fashion to its use in identifying microscopic inorganic particles in water (5,23).

The EM has been a great aid in brewing research and has great potential as an analytical tool in the future. The EM is no longer an instrument to be used solely for determining ultrastructure and now can be used more fully in microbiology, biochemistry, and physics as well. Through a marriage of disciplines, a more complete understanding of the yeast cell and of beer may be achieved.





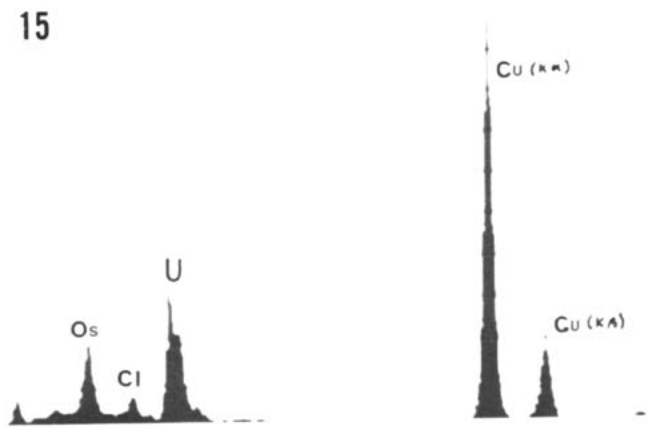


Fig. 13-15. *Pediococcus damnosus* var *II*. **Fig. 13.** Bright-field image of *P. damnosus* var *II* (fixed with 5% glutaraldehyde, 1% OsO_4 and stained with 2% uranyl acetate. Bar = 100 nm. **Fig. 14.** Elemental dot map for uranium in the same cell. Bar = 100 nm. **Fig. 15.** Energy-dispersive X-ray spectrum for the same cell collected for 300 sec in the transmission electron microscope mode using a 2.0 μm electron probe spot size. Copper peaks are from the copper grid, and the chlorine peak is from the embedding plastic.

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