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RESEARCH NOTE

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Scanning Electron Microscopy Studies of *Saccharomyces cerevisiae* Structural Changes by High Hydrostatic Pressure Treatment

Woo-Suk Bang* and Barry G. Swanson[1]

Department of Food Science, North Carolina State University, Raleigh, NC 27695, USA
[1]Department of Food Science and Human Nutrition, Washington State University, Pullman, WA 99164, USA

Abstract The structural change and leakage of cellular substances of *Saccharomyces cerevisiae* attributed by high hydrostatic pressure (HHP) treatment were observed with scanning electron microscopy (SEM). *S. cerevisiae* (ATCC16664) was inoculated in apple juice for 10 min at 23°C and the apple juice treated at 138, 207, 276, 345, and 414 MPa pressure for 30 sec at 23°C. Increased of roughness, elongation, wrinkling, and pores on yeast cell surfaces, the yeast cell walls were severely damaged by HHP treatment from 276 to 414 MPa. Inactivation of *S. cerevisiae* by HHP is dependent on structural changes on the cell walls observed with SEM.

Keywords: high hydrostatic pressure (HHP), *Saccharomyces cerevisiae*, scanning electron microscopy (SEM)

Introduction

The objective of food preservation is inactivation of spoilage and pathogenic microorganisms to increase shelf life and retain the sensory qualities of processed foods. Pasteurization and sterilization are the most common methods of thermal processing. Thermal processing affects not only inactivation of microorganisms, but also enzymes and other proteins leading to possible undesirable color, flavor, and texture. High hydrostatic pressure (HHP) is a potential nonthermal processing alternative to thermal processing. HHP processing inhibits spoilage and pathogenic microorganisms as well as enzymes retaining the 'fresh-like' qualities of foods, especially color and flavor (1-5). Rovere (6) stated the advantage of HHP food processing as: 1) no increase in temperature; 2) no waste products; and 3) minimal cooling water required. The effect of HHP treatment on structural changes and specific cell functions of bacteria were studied by Marquis (7) and Shimada *et al.* (8).

Molid (9) reported that HHP treatments affect biological molecules, protein denaturation, enzyme inactivation, genetic mechanisms, and biochemical reactions. The mechanisms of inhibition of microorganisms are difficult to explain because the inactivation of microorganisms by high pressure treatments includes wide range of pressure magnitudes. For example, 5 $\log_{10}$ CFU/mL of *Clostridium botulinum* was inactivated by 900 MPa for 4 min at 110°C in buffer (10) whereas a treatment of 241 MPa for 2 min at 28°C inactivated 5 $\log_{10}$ CFU/g in the level of *Vibrio vulnificus* in oysters (11).

Even though the mechanism of microbiological inactivation with HHP treatment is not well known, membrane permeability is the most acceptable theory (12). The other theory is the disappearance of ribosome bodies of *Saccharomyces cerevisiae* cell during HHP treatments (13,14). Harrison *et al.* (15) reported that disruption of *S. cerevisiae* cellular organelles and absence of ribosome bodies provided evidence to support these mechanisms of yeast inactivation. Shimada *et al.* (8) reported that the membranes of *S. cerevisiae* cells were disrupted and intracellular substances were released during HHP treatment (8,16,17). The objective of this study was to observe the morphological change of *S. cerevisiae* cells before and after HHP treatments.

*Corresponding author: Tel: +1-919-513-4600; Fax: +1-919-515-7124
E-mail: wbang@yahoo.com
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Materials and Methods

***S. cerevisiae* preparation** *Saccharomyces cerevisiae* (ATCC 1664; Rockville, MD, USA) cells were cultured in a 200-mL Erlenmeyer flask. The flask containing 50 mL of yeast malt broth (Difco, Sparks, MD, USA) was incubated at 225 rotations per min (rpm) in a controlled temperature orbital water bath shaker (Model MSB-3322A-I; GS Blue Electric, Blue Island, IL, USA) for 7 hr at 25°C. A 20 mL suspension of *S. cerevisiae* cells were transferred into a 1,000-mL Erlenmeyer flask containing 500 mL of yeast malt broth (YMB) and incubated the flask in the controlled temperature orbital water bath shaker at 225 rpm for 17 hr at 25°C. Four 100 mL suspensions of *S. cerevisiae* cells were transferred into four 1,000-mL Erlenmeyer flasks containing 400 mL of YMB, incubated in a controlled temperature orbital water bath shaker at 225 rpm for 9 hr at 25°C, and placed in an ice water bath for 15 min. Approximately 125 mL of the chilled yeast suspensions were distributed to sterile 250-mL centrifuge bottles and centrifuged (11,950×g, 5 min, Model J2-HS; Beckman, Palo Alto, CA, USA) for 10 min at 10°C. The supernatants were discarded. The pellets were suspended in 100 mL of YMB and centrifuged (11,950×g, 10 min) again at 10°C. The supernatants were discarded. The pellet was suspended in a 200 mL volume with YMB. A 5 mL aliquot of yeast cell suspension was transferred into 15-mL disposable

conical centrifuge tubes and centrifuged (11,950×g, 5 min) at 4°C. After centrifuging, the supernatants were discarded. The pellets were resuspened with 1 mL of sterile 20% glycerol and the conical centrifuge tubes and stored at –20°C.

High hydrostatic pressure (HHP) treatment A frozen pellet of *S. cerevisiae* was thawed by placing the centrifuge conical tube in cold water (10°C) for 5 min. and centrifuged (11,950×g, 5 min) at 4°C. The glycerol was discarded. The pellet of *S. cerevisiae* was inoculated into 1,000 mL apple juice and incubated for 10 min at room temperature. Apple juice was purchased from local market. Sterile polyethylene pouches (4 mil, 5.3×12 cm, Power Plastics Inc., Paterson, NJ, USA) were filled with 100 mL of the apple juice suspension and heat sealed with a TISH-3 type sealer (E-Z Aduit Bankpak Inc., Baltimore, MD, USA) at scale 3.5.

The apple juice suspensions were maintained in an ice water bath for resuscitation and placed in the pressure vessel of a HHP system (Engineered Pressure Systems, Inc., National Forge Co., Wilmington, MA, USA). The HHP system includes a pressure vessel, transferring yoke, pump, and temperature control systems. The capacity of pressure is 689.5 MPa. The dimensions of the inside cylinder pressure vessel are 4 inches in diameter and 10 inches in depths. The pressure vessel was filled with 5% Mobil Hydrosol 78 aqueous solution (Mobil Oil Crop, NY, USA) to transmit pressure.

When the pump is started, the intensifier of the pump system is driven to increase pressure to a selected set point.

The pump is stopped and the timer is started when the desired pressure is attained. When the set timer reaches, the control system sends a signal for the decompression, and the Mobil Hydrosol 78 solution goes through capacity tubes and returns to the pressure medium tank. Duplicated HHP treatments were performed at pressure 138, 207, 276, 345, and 414 MPa for each 30 sec. The come-up times to reach the selected pressures were approximately 90, 130, 160, 185, or 215 sec at the HHP of 138, 207, 276, 345, or 414 MPa, respectively. The decompression time was less than 10 sec. HHP operation times exclusively defined the holding time the selected pressure magnitude.

Viability of *S. cerevisiae* The viability of *S. cerevisiae* before and after HHP treatment was reported as colony forming units (CFU/mL) on potato dextrose agar (PDA) plates. One mL of apple juice containing the *S. cerevisiae* suspension was taken before and after HHP treatment and serially diluted with 99 mL of 0.1% sterile peptone solution. Two plates were used for each dilution and were incubated for 72 hr at room temperature. The viable counts between 25 and 250 were calculated from the mean of the 2 plates.

Scanning electron microscopy (SEM) The HHP treated *S. cerevisiae* in apple juice was transferred to 15-mL sterile plastic disposable conical test tube and centrifuged (11,950×g, 5 min) at 4°C. The supernatant was discarded. The yeast pellets were fixed in 2% paraformaldehyde (PFA), and 2.5% gluraraldehyde (PIPES) buffer at pH 7.2 for 16 hr at 23°C. After rinsing in PIPE buffer, the yeast cells were post-fixed in 2% osmium (OsO_4) for 2 hr at 23°C. The yeast cells were dehydrated in an acetone series of 30, 50, 60, 70, 80, 90, 95, and 100% at each 10 min intervals. The yeast cells were further dried in 100% acetone for 30 min at 23°C and transferred to 100% hexamethyldisilazane (HMDS) for additional 30 min at 23°C. The dehydrated yeast cells were placed on carbon tape before attachment to aluminum stub, coated with gold in a Hummer V Technics Sputter Coater (Technics Inc., Alexandria, VI, USA) to a thickness of >200 Å, and observed with a SEM (S570; Hitachi, Tokyo, Japan) at 20 Kv. The camera was used type 55 positive/negative Polaroid film.

Results and Discussion

Viability of *S. cerevisiae* before and after HHP treatment The initial *S. cerevisiae* population was 6 $\log_{10}$ CFU/mL. Figure 1 described as inactivation of *S. cerevisiae* at HHP treatment of 138, 207, 276, 345, or 414 MPa for 30 sec at 23°C. When the pressure magnitude increased, the inactivation of *S. cerevisiae* increased. Four $\log_{10}$ CFU/mL of *S. cerevisiae* were inactivated by a HHP treatment of 276 MPa for 30 sec at 23°C. The inactivation of *S. cerevisiae* was dependent on the pressure. Pandya *et al.* (18) reported no inactivation of *S. cerevisiae* in citric acid buffer at pH 4 by a HHP treatment of 151.7 MPa for 10 min at 25°C. When the pressure magnitude was increased to 202.7 MPa, 2 $\log_{10}$ CFU/mL of *S. cerevisiae* were inactivated. That is, increasing pressure magnitude increased inactivation of microorganisms. Six $\log_{10}$ CFU/mL of *S. cerevisiae* were inactivated by a HHP treatment of 345 MPa for 30 sec at 23°C. Similar increases in the magnitude of pressure increased inactivation of *Lactobacillus dellbrueckii* ssp. *bulgaricus* (17) and *Escherichia coli* (19).

SEM of *S. cerevisiae* before and after HHP treatment Figure 2a presented a typical *S. cerevisiae* cell. *S. cerevisiae* exhibits an oval shape and smooth cell wall surface. After *S. cerevisiae* was inoculated into apple juice for 10 min at room temperature (23°C), observed morphological change of *S. cerevisiae* cells were little. One or two bud scars were observed (Fig. 2b). A bud scar is the relic of a previous bud site on a mother cell. After the 138

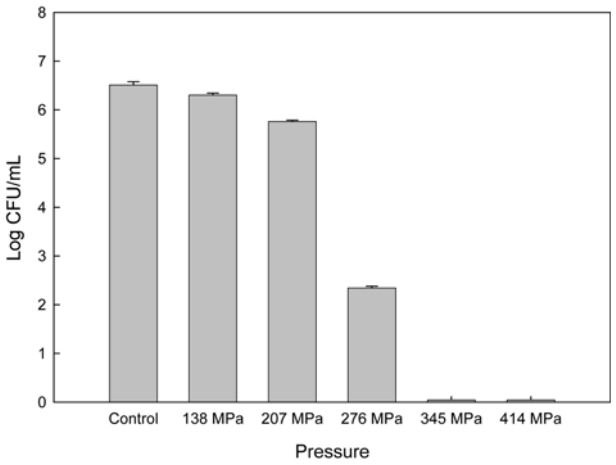

Fig. 1. Survival of *S. cerevisiae* after selected HHP treatments for 30 sec at 23°C in apple juice.

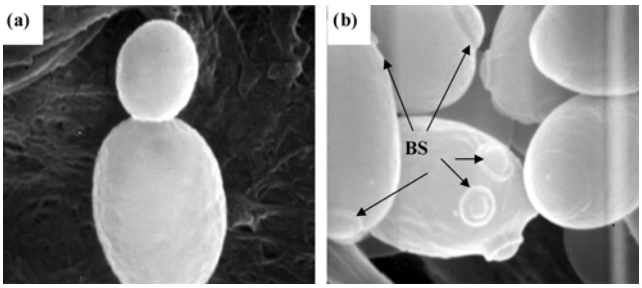

Fig. 2. Scanning electron microscope (SEM) photograph (×12 K). Untreated *S. cerevisiae* (a) and *S. cerevisiae* (b) was incubated in apple juice for 10 min at 23°C in apple juice. Bud scars are indicated by BS.

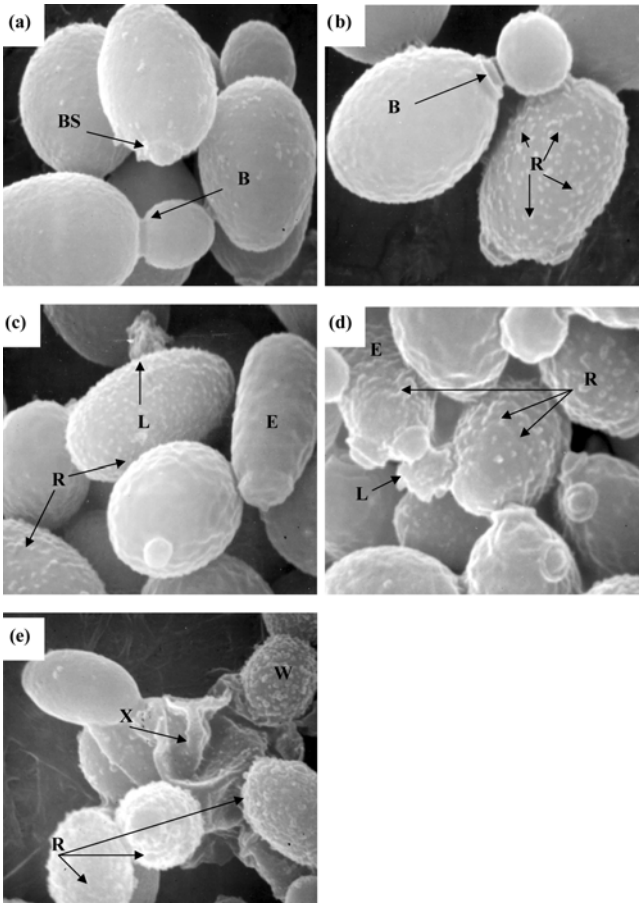

Fig. 3. SEM photograph (×12 K) of (a) 138, (b) 207, (c) 276, (d) 345, and (e) 414 MPa HHP treated *S. cerevisiae* for 30 sec at 23°C in apple juice. Bud scars, budding, surface roughness, elongated, leakage substances, shrinking, winkled, or exploded cells are indicated by BS, B, R, E, L, S, W, or X, respectively.

and 207 MPa pressure treatments of *S. cerevisiae* for 30 sec at 23°C, *S. cerevisiae* cell surface changed slightly. Roughness increased on the cell surface (Fig. 3a and 3b), but the shape of the cell was not changed. The 207 MPa HHP treated *S. cerevisiae* exhibited on rougher surfaces than the 138 MPa HHP treated *S. cerevisiae* cells. Budding was observed between a mother and daughter cell of *S. cerevisiae* at the bud scar (Fig. 3b). During the budding process, the cell from which the bud will develop is termed the mother cell, while the cell being formed by the bud is termed the daughter cell. HHP treatment did not impact to budding process, which was typical yeast reproduce.

Cell surfaces of 276 MPa HHP treated *S. cerevisiae* were more severely damaged than those of the 207 MPa HHP treated *S. cerevisiae* (Fig. 3c). The 345 MPa HHP treated *S. cerevisiae* cells presented increased surface roughening, elongation, and leakage of intracellular organelles (Fig. 3d). Shimada *et al.* (8) reported that intracellular organelle of *S. cerevisiae* cells were released by a pressure treatment of 300 MPa for 10 min at 25°C. The exposed intracellular organelles were difficult to find after the HHP treatment of 276 MPa. The complex alterations, surface roughness, wrinkle, and large pores were observed on *S. cerevisiae* cell surfaces after a 414 MPa treatment for 30 sec at 23°C (Fig. 3e). Weng (17) reported that cell disruption and large pores were observed after 414 MPa treated *L. delbrueckii* ssp. *bulgaricus*. Also, large pore formation in yeast cells was attributed to a HHP treatment of 500 MPa for 10 min at 25°C (9). Therefore, cell disruption is a potential mechanism for inactivation of *S. cerevisiae* by HHP treatment.

The structural changes of *S. cerevisiae* observed by SEM include increasing roughness of cell surfaces, cell elongation, surface wrinkling, and pores on cell surfaces. The inactivation and disruption of *S. cerevisiae* cells were increased when the intensity of pressure increased from 138 to 414 MPa. Also, the frequency of observed changes in *S. cerevisiae* increased when the pressure magnitude increased. Six $\log_{10}$ CFU/mL of *S. cerevisiae* in apple juice were inactivated by a pressure treatment of 345 MPa for 30 sec at 23°C.

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