Structural Changes and Inactivation of Saccharomyces cerevisiae in Grape Juice Induced by High Hydrostatic Pressure*

초고압에 의한 포도주스의 *Saccharomyces cerevisiae* 구조적 변화와 사멸효과

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목 차

<국문요약>

포도는 전세계에서 널리 소비되는 과실로 포도 과피에 존재하는 천연색소인 flavonoid는 혈중 콜레스테롤 함량 저하, 항알러지성, 항암성, 항바이러스성, 항염성의 생리적 기능이 있다고 알려져 있다. 최근에 들어와 이들 과실주스 가공에 열처리를 최소화하는 살균방법으로 자연 그대로의 영양성분, 맛과 향기 개선을 위한 초고압 처리에 관한 연구가 폭넓게 이루어지고 있다. 본 연구는 주스에서 문제가 되고 있는 ethanolic spoilage균주인 *S. cerevisiae* 의 초고압 살균 효과와 세포 구조적 형태를 연구하였다. 1.2×10^6 cfu/ml의 *S. cerevisiae*를 포도주스에 접종하고 24시간 배양하여 멸균한 high barrier주머니에 20ml씩 넣고 20℃에서 200-600 MPa 조건으로 0-20분 동안 초고압 장치로 실시하였다. 생균수는 YM agar로 poured 방법으로 실시하였으며 200 MPa에서 5, 10, 15, 20분 후의 생균수는 각각 2.2×10^7, 4.5×10^4, 2.8×10^4, 9.8×10^3, 9.5×10^3 cfu/ml로 tailing 현상을 관찰하였고, 400 MPa에서 5분 후 급격하게 감소하였다. *S. cerevisiae* 의 사멸속도는 초고압 처리가 높을수록 증가했으며 세포 손상도는 압력과 처리시간이 길수록 증가하였다. 이들 조건에 따른 효모 세포의 구조적 관찰을 scanning electron microscopy와 transmission electron microscopy로 하였다. *S. cerevisiae* 세포는 압력에 의한 pinhole, surface roughening을 발견하였고, 세포 내부의 세포질, 액포, 핵 손상과 세포질 물질들이 압력에 의하여 세포벽으로 이동하여 내부가 비어있는 현상을 관찰하였다.

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I. Introduction

New technologies such as non-thermal physical treatment processing are being developed to inactivate spoilage and pathogenic microorganisms. Traditional thermal processing, including pasteurization and retorting, can result in adverse changes to the flavor, taste, and nutrient content of foods. High pressure processing has been investigated as a method to stabilize citrus juices at reduced temperatures by inactivating the native microflora (1,3,7,13,16,18,22,23,27,31,34). The technology appears to successfully eliminate native microorganisms capable of causing spoilage in refrigerated citrus juices. Microbial decomposition of fruit juices is most frequently associated with fermentative yeasts such *S. cerevisiae* which causes ethanolic spoilage, carbonation, production of hydrogen sulfide, and other off-odor (14,17). Since fruits are relatively low in protein and starch, there have been no sensory quality changes resulting from protein denaturation and the swelling of starch mixtures caused by exposure to high pressure. Successful inactivation of microorganisms by high hydrostatic pressure has been reported through numerous researchers (2-18). Many factors are reported to influence the microbial inactivation such as effective pressure strength and duration (1,8), temperature of treated food (28), and enzymatic inactivation of treated foods (5,12,16,21,26,29,32).

The physical phenomenon responsible for microbiological inactivation is less clear. The most popular theory describing pressure inactivation of microorganism was recently reviewed by Castro *et al.* (3), in which pore formation and destruction of the semipermeable membrane barrier are described. Changes to the *S. cerevisiae* cells observed by scanning electron microscopy (SEM) after pressure treatment, showing surface roughening and pinholes (25,30), along with craters or holes, elongation, roughening, wrinkling, and increased bud scare formation (24,25). Reports on transmission electron microscopy (TEM) techniques to observe high pressure-treated yeast cells are scarce. TEM methodologies were previously utilized to observe budding and cell wall rupture (31) of *S. cerevisiae* in studies not involving high pressure. Thus, the objective of this study was to utilize SEM and TEM to observe structural changes of *S. cerevisiae* with high hydrostatic pressure.

II. Materials and Methods

1. High pressure equipment

A Cold Isostatic Press manufactured by ABB Autoclave (Quintus Food Processor 6, Columbus, OH, USA), consisting of an 800 milliliters pressure vessel, a piston-type intensifier, and an external hydraulic pump, was used. A water jacket was installed around the pressure vessel, and the temperature of the pressure medium (water) was maintained at 20°C. A thermocouple was placed inside the vessel to measure the temperature of the pressure medium during high pressure treatment.

2. Test strains

The strains of *S. cerevisiae* (ATCC 166664, Rockville, MD, USA) yeast cells were cultured in yeast malt broth (DIFCO 0711-01-9, Detroit, MI, USA) at 25°C.

3. Preparation of medium

Reactivated *S. cerevisiae* cell was inoculated at $2x10^6$ cfu/ml in grape juice (pH 3.4, 12% sucrose)

and incubated for 2 days at 25°C

4. High pressure treatment

Polyethylene bags were washed using 2% (w/w) H_2O_2 solution and dried in a sterilized clean bench under UV light. Twenty milliliters of grape juice medium was transferred into the polyethylene bags (45 mm x 120 mm) and the bags were then heat-sealed without entrapping air bubbles. The bags were put into the pressure vessel and pressurized at 200-600 MPa for 1-20 min at 20°C. After high pressure treatment, the bags were removed and held in ice-water for further analysis.

5. Enumeration of survivors

The high pressure-treated samples were kept at 4°C. to minimize microbial growth. The survivor cell counts were determined by plating 1 ml of diluted samples onto duplicate plates of YM agar (Difco Laboratory, Detroit, MI, USA). Colonies were counted after 2 days of incubation at 25°C.

6. Electron microscope observation

SEM and TEM were performed on the microbial suspensions after high pressure treatment. For SEM observation, samples were treated, fixed, and dehydrated following the same method as for both SEM and TEM observations. Critical point drying of the yeast cells were accomplished by washing three times with 100% hexamethyldisilazane (HMDS) for 15 min. The gold-coated sample was observed with a JSM 5410-LV SEM (JEOL, Tokyo, Japan). For TEM, both untreated and treated cells of *S. cerevisiae* were centrifuged at 4000×*g* for 10 min at 10°C, and the supernatant was discarded. The cells were resuspended in 2 ml of 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) and fixed for 24 h at 4°C. The microcentrifuge tube contents were centrifuged at a constant 10,000×*g* for 20 s in Beckman Model Microfuge E (Pals Altto, CA, USA).

The supernatant was discarded, and the pellet was resuspended in 0.05 M sodium cacodylate buffer (pH 7.4). The 1 mm^3 yeast cell cubes were infiltrated in 10 ml Spurr resin, and the resin was replaced every 2 h for 24 h on a 5 rpm rotator titled at a 45 angle. Polymerization of Spurrs resin to form specimen blocks was induced heating the resin in molds at 70°C. for 8 h. The specimen blocks formed were hand trimmed with a razor blade and sectioned into a thickness of approximately 0.5μm with a Reichert Model OM2(Austria) ultra microtome. The sections were stained with 2% uranyl acetate and Reynolds lead citrate, and viewed with JEM 1010 TEM (JEOL, Tokyo, Japan) transmission electron microscope operated at 80 kV.

III. Results and Discussion

1. High pressure inactivation of Saccharomyces cerevisiae

The *S. cerevisiae* population was reduced from an initial concentration of 2.2×10^7 cfu/ml to total inactivation. Figure 1 shows the effect of high pressure on inactivation of *S. cerevisiae*. Under high pressure at 200 MPa, the number of survivors was reduced to 4.5×10^4 cfu/ml for 5 min. However *S. cerevisiae* suspended in grape juice was completely inactivated at 400 MPa. In the application of high pressure processing, which is a batch type process, the duration of treatment exerts a critical influence on the cost. The duration of high pressure treatment in the following experiments was limited to less

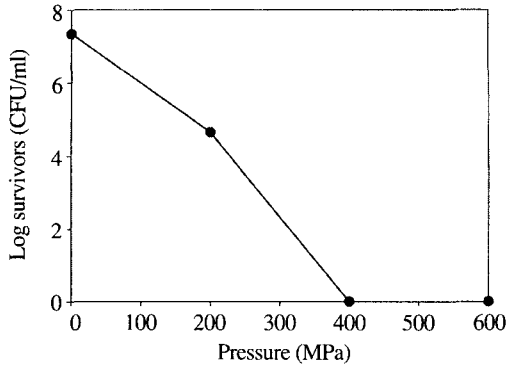

Fig. 1. Effect of high pressure on inactivation of *S. cerevisiae*. The pressure was treated for 5 min at 20°C

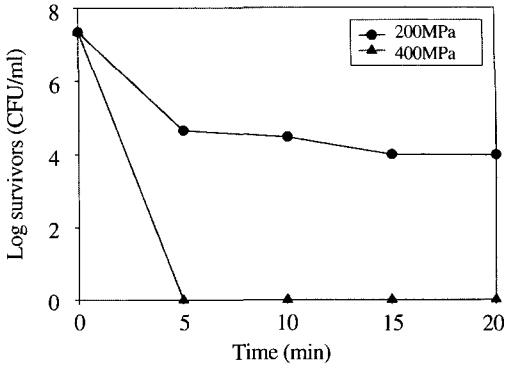

Fig. 2. Survival curve of *S. cerevisiae* at different pressure. The pressure was treated at 20°C

than 20 min (Fig. 2). The survivor cell number of *S. cerevisiae* suspended in grape juice was reduced to 10^3 cfu/ml for 5 min and "tailing" phenomenon was observed at 200 MPa. However, a further reduction completely inactivated the cells at 400 MPa and 20°C. These results were similar to the inactivation in *S. cerevisiae* reported by Ogawa *et al.* (21). They showed that the reduction of the total microflora in Satsuma mandarin orange juice by 5 log CFU/ml required pressure treatments of 350 MPa for 30 min or 400 MPa for 5 min.

2. Changes in the ultrastructure of S. cerevisiae by high pressure treatment

Untreated (control) *S. cerevisiae* cells inoculated in sterile grape juice for 20 min and viewed with TEM exhibited cellular organelles including the nuclei, vacuoles, and cytoplasmic materials (Fig. 3-A). After pressure treatment of *S. cerevisiae* inoculated in grape juice, the micrograph of yeast cell structure exhibited cytoplasmic shrinkage, cytoplasmic compartmentalization, and cellular organelle disruption (Figs. 4-B, C, and D).

Harrison *et al.* (14) showed after high voltage pulsed electric fields (PEF) treatment of *S. cerevisiae* inoculated in apple juice, the cytoplasmic material parted from the cell wall and cell wall voids. Shin *et al.* (30) also observed that PEF treatment in Lactobacillus plantarum shrinks the cytoplasmic membrane away from the outer membrane, and the blank space separated inner materials from the membrane.

In our observations, it was evident that the structural changes in yeast cells of cytoplasmic material shrinkage toward the cell wall and voided cytoplasmic are caused by the pressure. Osumi (24) reported on the inner structure of *S. cerevisiae* treated with hydrostatic pressures of 100, 200, 300, 400, and 500 MPa, and the apparition of nucleus was 14, 60, 81, 90, and 100%, respectively.

SEM micrographs of both the control and the pressurized cells are shown in Fig. 4. *S. cerevisiae* cells treated at pressures lower than 600 MPa at 20°C. showed slight effects on the outer shape, pinholes (Fig. 4-A), surface roughening (Fig. 4-C), and cell wall rupture (Fig. 4-D). Osumi *et al.* (25) reported that *S. cerevisiae* cells treated to pressure lower than 400 MPa at room temperature showed a slight effect on the outer shape, but the bud scar area of the cell wall disrupted and/or damaged was at pressure higher than 500 MPa. It is difficult to

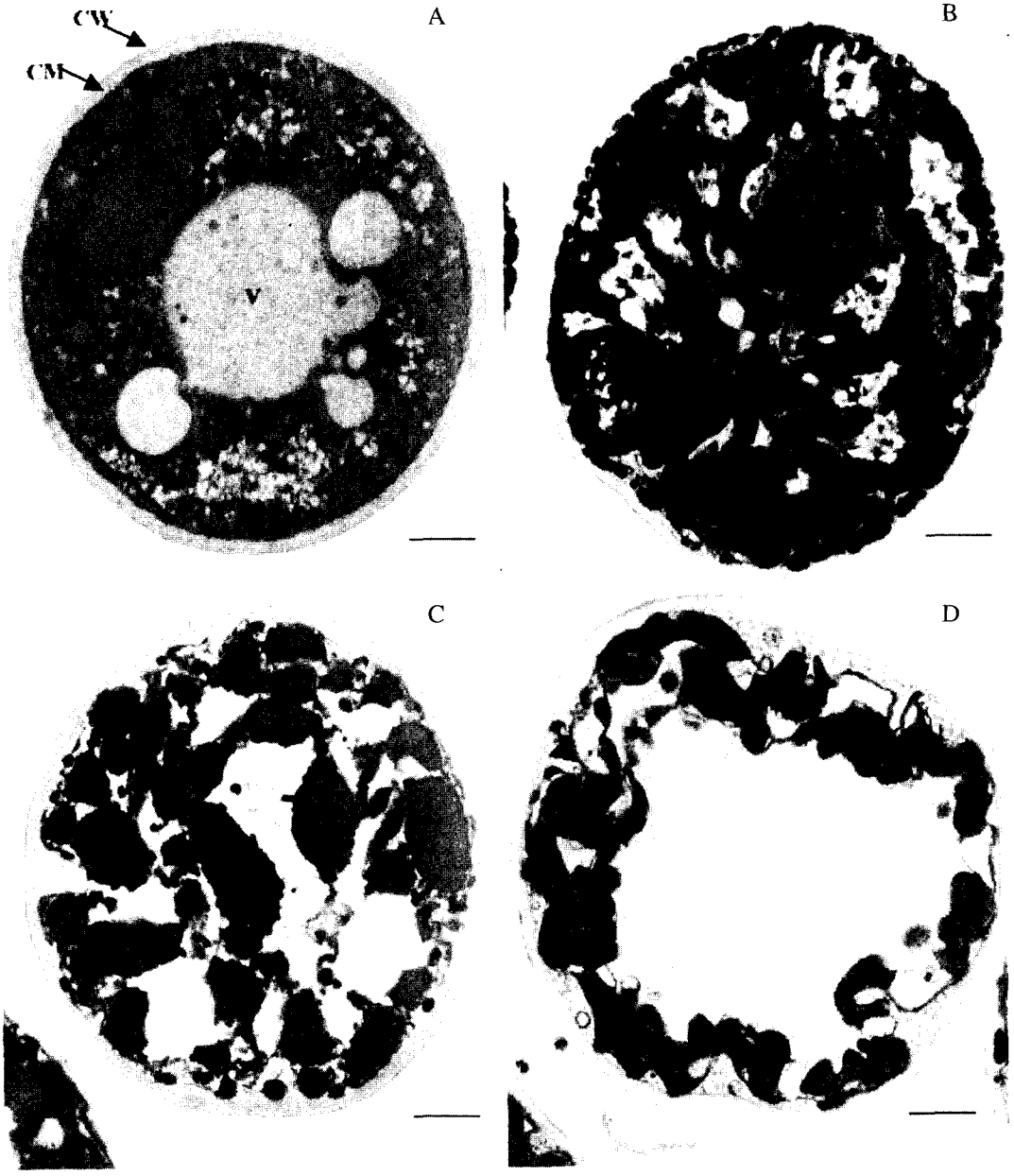

Fig. 3. Transmission electron microscope of high pressure treated *S. cerevisiae* in grape juice for 5 min at 20°C

(A) untreatment control cell, showing nucleus(N), nucleus membrane(NM), vacuole(V), cell membrane(CM) and cell wall(CW) ; (B) pressure-treated cell at 200MPa ; (C) 400MPa ; (D) 600MPa. Bar corresponds to 500nm (_____) .

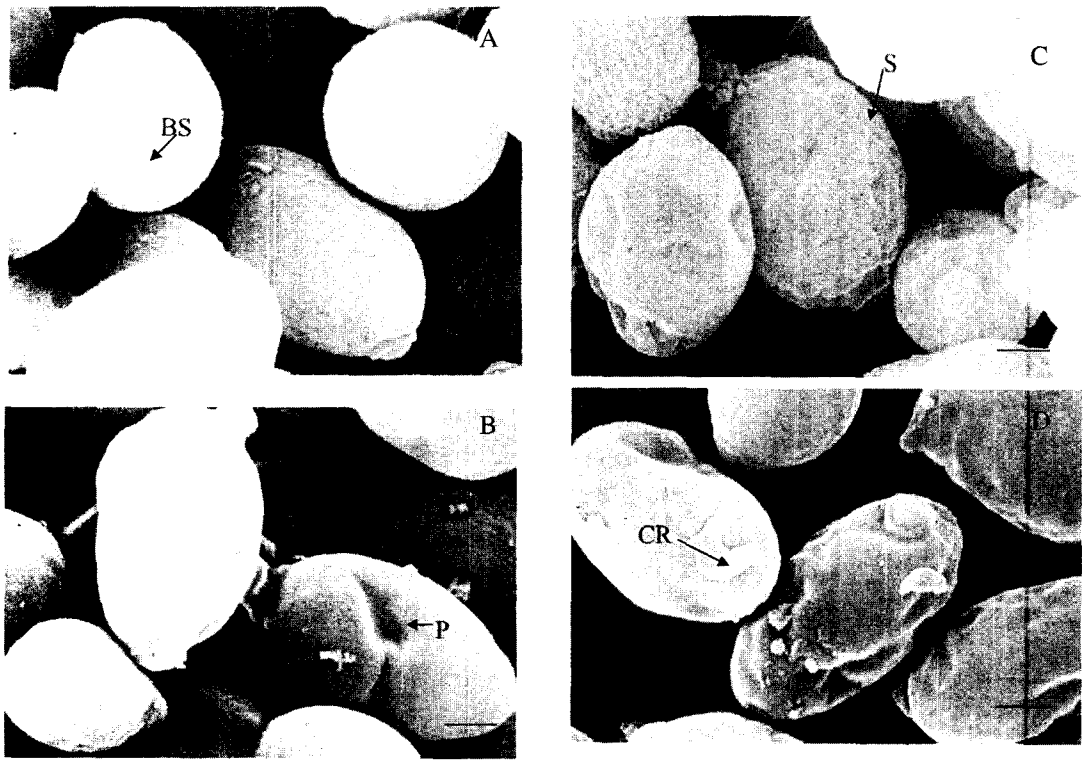

Fig. 4. Scanning electron microscope of high pressure treated *S. cerevisiae* in grape juice for 5 min at 20°C.
(A) untreatment control cell ; (B) pressure-treated cell at 200MPa ; (C) 400MPa ; (D) 600MPa.
BS: bud scare, P: pinholes, S: surface roughning and CR: cell wall rupture. Bar corresponds to 1μm (———).

identify the effects of high pressure inactivation because complex damage could be observed after the high pressure treatment. Sohn (31) predicted high pressure inactivation has two effects, one is the destruction of the cell structure by physical effect of high pressure such as adiabatic expansion during high pressure treatment and the other by protein denaturation under high pressure. From these observation it is suggested that the structural effects of hydrostatic pressure on *S. cerevisiae* occured on the membrane system particularly on the cell membrane invagination in the cytoplasm.

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