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Leakage of Cellular Materials from *Saccharomyces cerevisiae* by Ohmic Heating

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Abstract The ohmic heating of foods for sterilization provides a shorter come-up time compared to conventional thermal processes. The electric fields as well as the heat generated by ohmic heating facilitate germicidal effects. In the present study, the effect of ohmic heating on the structure and permeability of the cell membrane of yeast cells, *Saccharomyces cerevisae*, isolated from *Takju* (a traditional Korean rice-beer), was investigated. The ohmic heating was found to translocate intracellular protein materials out of the cell wall, and the amount of exuded protein increased significantly as the electric field increased from 10 to 20 V/cm. As higher frequencies were applied, more materials were exuded. Compared to conventional heating, more amounts of proteins and nucleic acids were exuded when these cells were treated with ohmic heating. The molecular weights of the major exuded proteins ranged from 14 kDa to 18 kDa, as analyzed by Tricine-SDS PAGE. A TEM study also confirmed the leakage of cellular materials, thus indicating irreversible damage to the cell wall by ohmic heating. It was, therefore, concluded that the electric fields generated by ohmic heating induced electroporation, causing irreversible damage to the yeast cell wall and promoting the translocation of intracellular materials.

Key words: Ohmic heating, *Saccharomyces cerevisiae*, permeability, electroporation, cell wall

It is generally known that electric field strengths or microwaves have specific physical, chemical, and biological effects on the biomaterials of cells. In the food industry, Anderson and Finklestein [2] initially examined milk sterilization with electric fields. Electrical and microwave applications not only inactivate microbial activity, but also reduce deterioration of food products caused by excessive conventional heating. Representative technologies for these protocols include a high voltage pulsed electric field process, low voltage electrical processing, and microwave heating. The mechanism of such heating methods is due to the internal generation of heat by electrical currents. The advantages of internal heating include higher efficiency and shorter heating time than conventional heating. It has been reported that the bactericidal effects of microwave heating are greater than those of conventional heating [10]. However, inactivation of microorganisms with a high voltage electric field disrupts the cell membrane due to electrohydraulic shock and electroporation [1, 9, 10, 20]. According to Cho *et al.* [4], the thermal effect is the main mechanism underlying inactivation of microorganisms by ohmic heating, and the thermal death kinetics are not significantly different among ohmic heating and conventional heating. In the inactivation kinetics of *Bacillus subtilis* spores, the decimal reduction time (D value) of those groups treated by ohmic heating was much smaller than that with conventional heating, plus electricity influenced the death rate of *B. subtilis* spores [4].

The heat transfer from ohmic heating can be applied to both sterilization and fermentation processes. It has earlier been reported that an electric current is capable of reducing the lag phase and inhibiting the latter growth phase of microorganisms [5]. Also, there have been many earlier reports on the rapid inactivation of microorganisms by ohmic heating compared to conventional heating, probably due to the fast heating rate and electrical effects of ohmic heating in addition to its thermal effects [7, 14, 16, 17]. Therefore, we have investigated the relationship between electric field strengths and their effect on microorganisms. Using yeast cells as an example, the concentration of exuded intracellular materials was compared with various electric field strengths of ohmic heating and also compared to conventional heating. Additionally, morphological changes of yeast cells and the inactivation mechanism during ohmic heating were investigated.

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MATERIALS AND METHODS

Experimental Instruments

The ohmic heating instruments consisted of a signal generator (Kenwood, AG-204, Japan), power amplifier (NF Electronics, 4205, Japan), data logger (Fluke data acquisition unit, Fluke Co., WA, U.S.A.), computer, and heating units. The heating unit was composed of titanium (Ti) electrodes for contact with the sample and a t-type thermocouple enclosed in an acryl amide box. The AC waves adapted to the ohmic heating system were observed and measured using a digital oscilloscope (Fluke combiscope, PM 3380A, Holland).

The conventional heating was performed in a water bath by sealing 1 ml of the prepared sample in a glass capillary, 10 cm in length and 0.45 cm in diameter. The rate of temperature increase was set at 0.5°C/sec to equal ohmic heating of 20 V/cm.

Preparation of Yeast

The *Saccharomyces cerevisiae* was isolated from *Takju*, Korean rice-beer, as described previously [12, 13]. The culture was suspended in a YM broth (Difco, Detroit, MI, U.S.A.) containing 20% glycerol (Sigma Chemical Co., St. Louis, MO, U.S.A.), and stored at −70°C. Before use, the culture was inoculated in the YM broth at 1% concentration, incubated at 27°C for 48 h, and then centrifuged at 1,000 ×g. The yeast cells were washed with 0.02 M phosphate buffer (pH 7.0) and then adjusted to 10% yeast suspension in the phosphate buffer. A 20 ml volume of this suspension was taken and treated with either ohmic heating at various voltages (10 V/cm, 15 V/cm, and 20 V/cm) and frequencies (60 Hz, 600 Hz, 6 kHz, and 60 kHz) or conventional heating.

Yeast Survival Rate

The survival rate of the yeast treated by ohmic and conventional heating was determined by measuring the ability to form colony on a potato dextrose agar (PDA) medium (Difco, Detroit, MI, U.S.A.). Immediately after treatment with a temperature range of 25°C to 90°C, the samples were diluted in 0.85% saline, spread on the PDA medium, and incubated at 27°C for 36 h. The final cell number was expressed as the colony forming units per milliliter (cfu/ml).

Quantification of Proteins and Related Materials

The treated yeast suspension was centrifuged at 1,000 ×g, and the total protein and nucleic acid contents of the supernatant were estimated by measuring absorbencies at 280 nm and 260 nm, respectively. The precise quantification of the total protein was further carried out by the Bradford method [3] using a phenol reagent kit (Biorad Laboratory, CA, U.S.A.).

Determination of Molecular Weights

Polyacrylamide gel electrophoresis was carried out in 16.5% Tricine-SDS PAGE in a Mighty Small™ SE245 unit (Hoefer, CA, U.S.A.). The running buffer consisted of 0.2 M Tris-HCl (pH 8.9) at the anode and 0.1 M Tris-HCl (pH 8.25) with 0.1 M Tricine at the cathode. The supernatant of the yeast suspension treated with either ohmic or conventional heating was incubated for 30 min at 40°C in 4% SDS, 12% glycerol (w/v), 50 mM Tris, and 2% mercaptoethanol (v/v) and then 0.01% Serva blue G (Serva electrophoresis, Heidelberg, Germany) was used as the tracking dye. The loading volume of each sample was 20 µl. Ten microliters of protein markers (Life Technologies, Gibco BRL, U.K.) composed of ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18,400 Da), lysozyme (14,300 Da), bovine trypsin inhibitor (6,200 Da), and insulin (α and β chains) (3,000 Da) were used. The electrophoresis was initially run at 30 V for 1 h, then at 105 V for 16 h. The protein bands were fixed for 30 min in a solution containing 50% methanol and 10% acetic acid, and then stained with 0.025% Serva Blue G in 10% acetic acid for 1 h. The background destaining of the gel was performed by shaking the gel in 10% acetic acid for 2 h and the solution was changed every 30 min [18].

Electron Microscopic Observation of Yeast Cells

The centrifuged yeast pellets were washed four times with phosphate buffered saline at 4°C and fixed in 5% potassium permanganate ($KMnO_4$) at room temperature for 90 min. The samples were then dehydrated in a graded ethanol and acetone series, and embedded in Epon 812. Sections were cut with a diamond knife using a microtome (Sorval MT-2, Ultramicrotome, Japan) and then collected on collodion-coated, carbon stabilized, copper grids. Sections with 400 to 600 Å thickness were stained with lead hydroxide for 20 min, scanned, and photographed with a JEM 100-II electron microscope operated at 80 kV [8].

Statistical Analysis

All statistical analyses were performed by an analysis of the variance (ANOVA) between treatments using SPSS version 7.5 (SPSS Inc., IL, U.S.A.), and the results are expressed as means of triplicate of each experiment. Significance was noted only at a level of $p<0.05$.

RESULTS AND DISCUSSION

Death Curve

As seen in Fig. 1, the heating rate of both the ohmic and conventional heating was the same, reaching 100°C in about 500 sec. The destruction of yeast cells began at around 50°C. The ohmic heating accelerated the cell destruction at a low temperature. The difference in the yeast cell

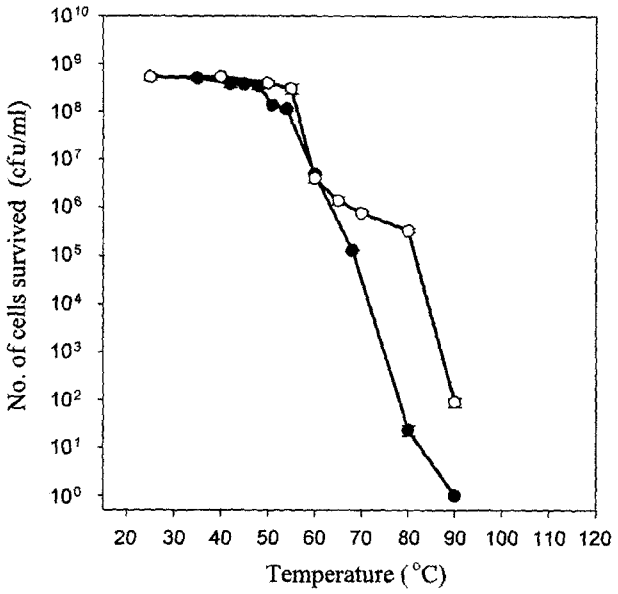

Fig. 1. Death curve of yeast with conventional water bath heating (○) and ohmic heating (20 V/cm) (●).

destruction rates between the ohmic and conventional heating became much pronounced at 70–80°C (Fig. 1).

The Effect of Electric Field Strengths

The electric field appeared to have both direct and indirect effects on the cell wall, and intracellular materials were exuded to the culture medium. These exudates seemed to be composed of amino acids, protein, nucleic acids, coenzymes, and related materials [19, 21]. As shown in Fig. 2, the absorbance at 260 nm of the nucleic acid was 2-fold ($p<0.01$) and the total protein content was 3-fold higher ($p<0.01$) with ohmic heating at 20 V/cm compared with that at 15V/cm. As the voltage increased from 10 V/cm to 20V/cm, the concentrations of nucleic acid and protein also increased. This is most likely due to the fact that the rate of temperature increase is associated with the voltage increased. Indeed, as shown in Fig. 2, as the voltage increased, the temperature rose more quickly and the exudation of the materials began earlier (Fig. 2).

Depending on the type of material treated, the efficiency of the frequency appears to be distinct. For example, the heat rate in Korean red pepper paste rapidly increased up to 5 kHz and decreased thereafter [6]. As for the yeast cells used in the current study, the frequency ranged from 60 Hz to 60 kHz and the heating rate increased accordingly. In the present study, the concentration of exuded intracellular protein was found to increase after 600 sec of treatment at 600 Hz, 6 kHz, and 60 kHz ($p< 0.01$), and this continued for longer time (900 sec) (Fig. 3). In contrast, the concentration of nucleic acid, increased when treated at 6 kHz or 60 kHz for 600 sec, was the same as that treated for 900 sec.

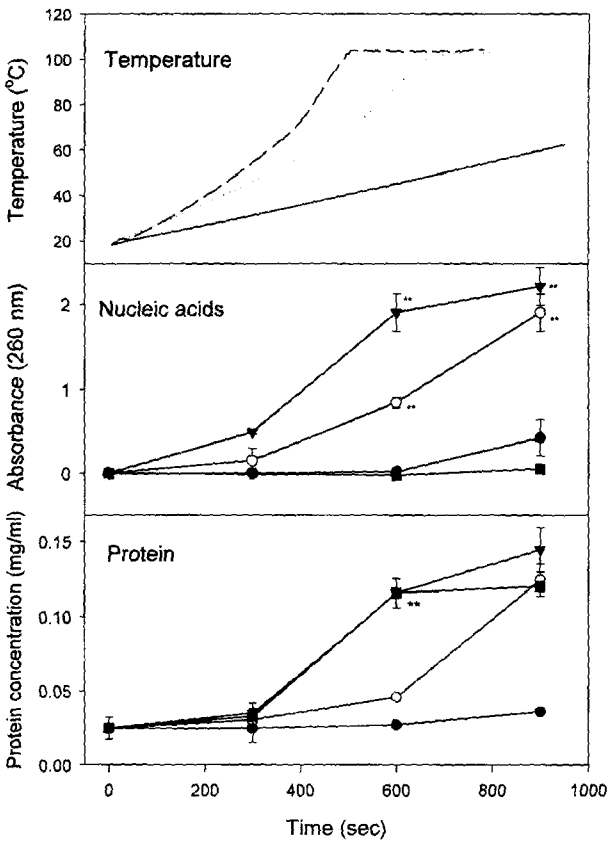

Fig. 2. Protein concentration, UV absorbency of nucleic acid, and temperature change in the supernatant of yeast suspension after ohmic heating at various electric field strengths (60 Hz, sine wave).

Values of nucleic acids absorbency and protein concentration are expressed as mean±SD. The temperature change is indicated by —— for 10 V/cm, ····· for 15 V/cm, and ------ for 20 V/cm. The nucleic acid absorbency and protein concentration are indicated by ● for 10 V/cm, ○ for 15 V/cm, ▼ for 20 V/cm, and ■ for conventional heating. The symbol ** means significant between 15 V/cm or 20 V/cm and 10V/cm at $p<0.05$.

Consistent with these results, it was also found that as the frequency increased from 60 Hz to 60 kHz, the rate of temperature increase remained the same. Thus, at all frequencies, 100°C was reached in approximately 600 sec and the exudation of nucleic acid and protein also began to increase at about 600 sec and continued to increase with a longer heating time (900 sec). As explained by Sastry (personal communication), at a low frequency, the electrical charge of the cells can readily accumulate, and thus the effect on cell permeability becomes stronger than at a high frequency, such a case as in microwave (Fig. 3). Accordingly, the above result suggested that with 6 kHz–60 kHz treatments, the yeast cells accumulated the electric field, thereby augmenting the permeability of the cell wall.

Thermal Effects

The translocation of intracellular materials due to ohmic heating has been suggested to be caused by the electrical or

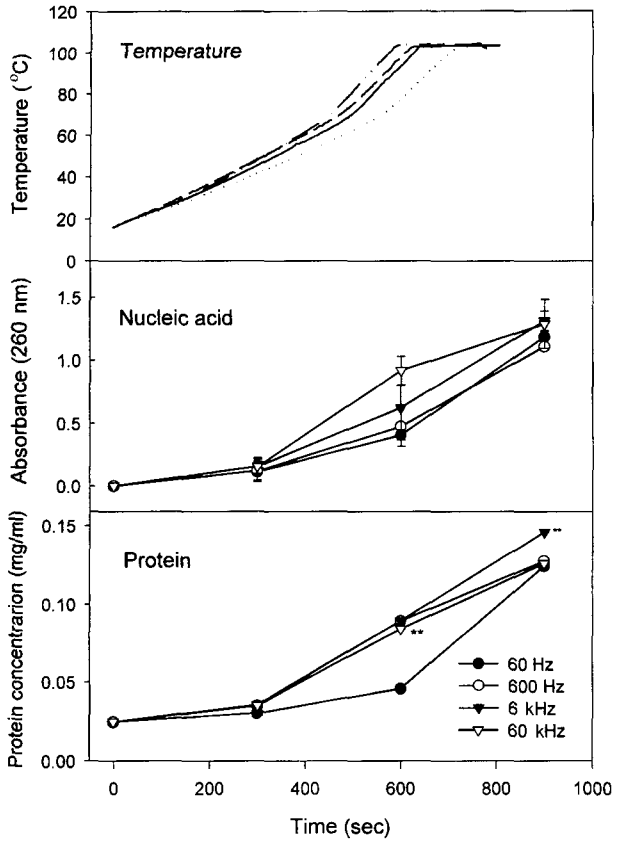

Fig. 3. Changes in protein concentration, UV absorbency of nucleic acid, and temperature change of yeast suspension with ohmic heating (15 V/cm) time at various frequencies.
Values of nucleic acids absorbency and protein concentration are expressed as mean±SD. The temperature change is indicated by —— for 60 Hz, ····· for 600 Hz, ------ for 6 kHz, and -·-·- for 60 kHz. The changes of nucleic acids absorbency and protein concentration are indicated by ● for 60 Hz, ○ for 600 Hz, ▼ for 6 kHz, and ▽ for 60 kHz. The symbol ** means significant between 60 Hz and 600 Hz, 6 kHz, or 60 kHz at $p<0.05$.

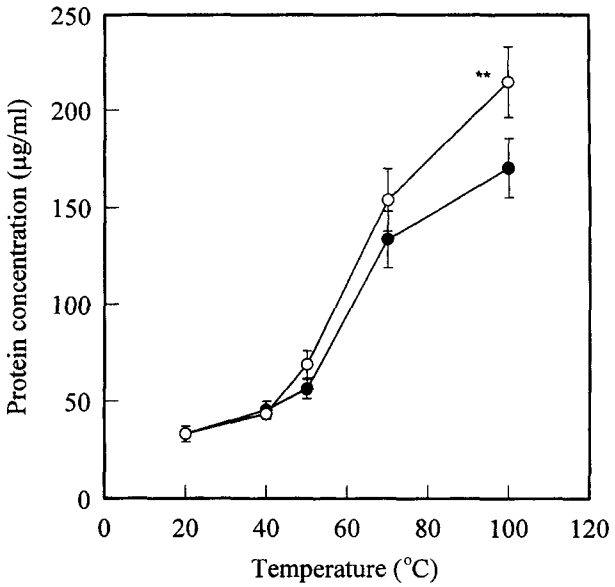

Fig. 4. Changes in protein UV absorbency of supernatant of yeast suspension treated by conventional heating (●) and ohmic heating (○) at 20 V/cm, 60 Hz.
The symbol ** means significant between conventional and ohmic heating.

thermal effects of rapid temperature increment [19]. Figure 4 shows the UV absorbance-temperature relationship of the yeast supernatant, when the time-temperature records for the ohmic and conventional heating were similar. The UV absorbencies for the ohmic and conventional heating groups were similar at below 50°C. However, at temperature above 50°C, the concentrations of exuded materials from the ohmic heated groups were higher than those from the conventional heating group ($p<0.01$). This result was in agreement with the destruction rate of the yeast cells, as shown in Fig. 1. As the yeast cell destruction started and the microbial destruction rate increased within a temperature range of 55°C to 60°C, the protein concentration in the exudate increased rapidly. The rate of protein exuded per unit temperature increase was found to be significantly higher ($p<0.01$) with the ohmic heating than the conventional heating. It is hypothesized that the higher exudation rate was not only dependent on the destruction rate of the yeast cells but also on the type of heating method. The influence of the electric field with the ohmic heating might have increased the rate of electroporation, thereby leading to excess exudation and cell death.

The Tricine SDS-PAGE method was used to investigate the molecular weight of the exuded proteins. As shown in Fig. 5, the amount of 15–16 kDa molecular weight protein increased with longer treatment time. The distribution of protein was more prominent in the exudates after the ohmic heating (lanes 1, 2, and 4 of Fig. 5) than after the conventional heating (lane 3). It should be noted that the cell membrane is composed of 40 different kinds of 15–16 kDa molecular weight proteins, and more than 60 different kinds of proteins with similar weight are found in the ribosome [15]. In the present study, most of the exuded proteins came from the cell membrane and a small part from ribosome. It would appear, therefore, that the ohmic heating resulted in alteration of the cell membrane causing leakage of proteins and the effect was stronger than the conventional heating. In conclusion, the difference was apparently due to the electrical field created by the ohmic heating.

Observation of Yeast Cells by TEM

Under a light microscope, the cell walls of the untreated, conventionally heated, and ohmic heated yeast cells showed little difference. However, when investigated using a transmission electron microscope (TEM), a morphological change in the cell membrane was seen in the treated yeast cells (Fig. 6): It was more intense after the ohmic heating (C) than the conventional heating (B). According to

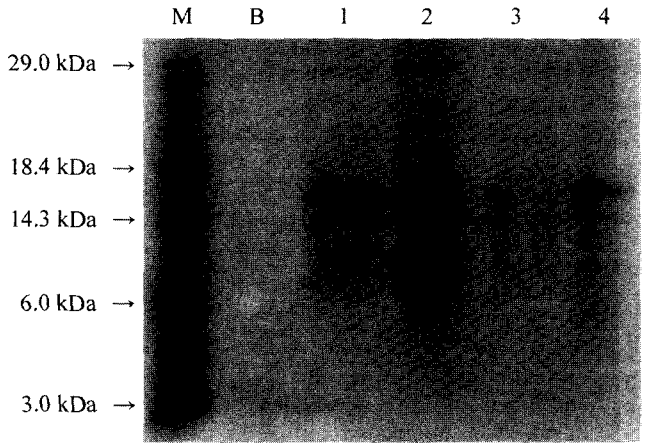

Fig. 5. Changes in Tricine SDS-PAGE patterns with different conditions of ohmic and conventional heating.
Lane **M** is the molecular weight marker; lane **B**, blank; lanes **1** and **2**, MW of extracellular protein after treatment by ohmic heating at 20 V/cm for 600 sec and 900 sec, respectively; lanes **3** and **4**, MW of extracellular proteins after treatment by conventional and ohmic heating at 10 V/cm for 900 sec, respectively.

Harrison *et al.* [11], when yeast cells are treated at 40 kV/cm for 5 to 15 sec, shrinkage of the cytoplasmic materials and detachment of the cell wall occur, and such changes are described as electroporation. In confirmation of Harrison's findings, it was found in the current study that the treated yeast cells also exhibited electroporation. Although no shrinkage of cytoplasmic materials was specifically identified, the development of space between the cell wall and the membrane was evident, based on small and irregular changes surrounding the cell wall (Fig. 6). In Harrison's study [18], cell debris was observed at a distance from the disturbed cells, whereas the current results found a regular distribution of cellular materials encircling the cells. As such, it would appear that the high electrical potential difference between inside and outside the cell wall caused a spontaneous direct disruption of the cell wall. At a low voltage, treatment for an extended period created a slow transmembrane electric potential (TEP) between inside and outside the cell wall, thus allowing regular and increased numbers of pore formation. Thereafter, the inside cell wall materials were released into the outside cell wall fluid, thereby inferring that the electroporation theory takes place with such phenomena. Corresponding to this theory, to study the bacterial growth kinetic, Cho *et al.* applied a low voltage to a cell culture and demonstrated shortening of the lag phase [5]. The movement of the intracellular materials was found to be continuous with both the ohmic and conventional heatings as compared to the untreated yeast cells, since concentrated debris still existed even when the cells were washed several times in the TEM process (Fig. 6). Therefore, it would appear that the electroporation caused by the electric field with ohmic heating increased the permeability of the cell wall.

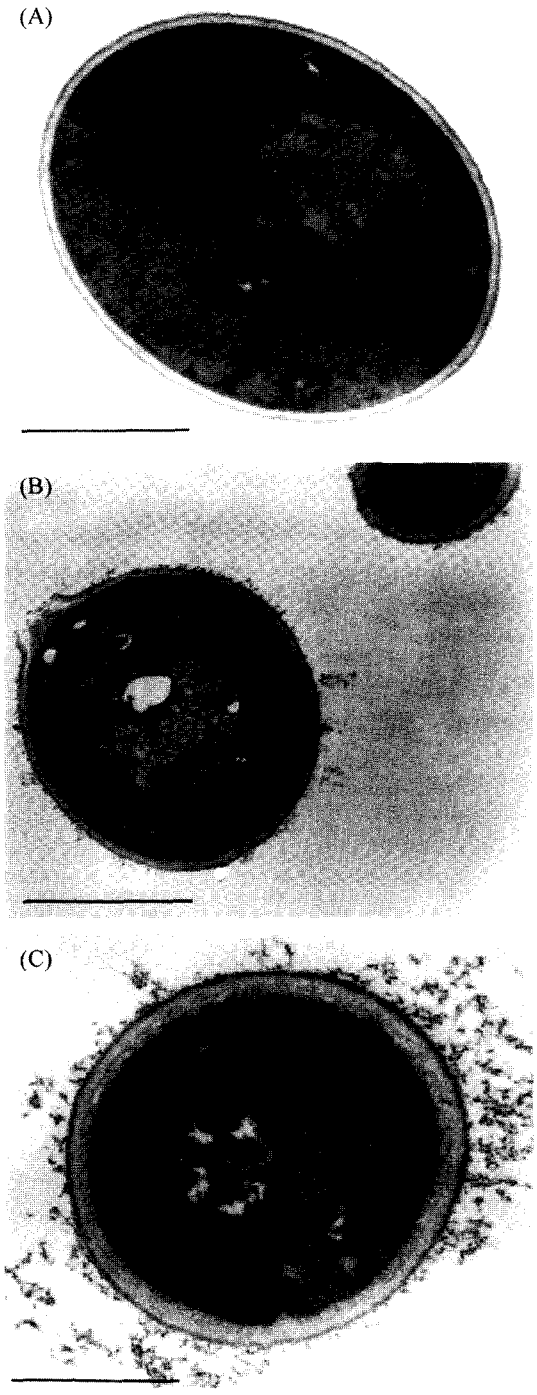

Fig. 6. Transformation of yeast cell membrane.
Photomicrographs show nontreated (A), conventional heated (900 sec) (B), and ohmic heated (20 V/cm, 900 sec) (C) yeast cells. The bars in A, B, and C correspond to 5 μm.

CONCLUSION

The current investigation found that the mechanism of microorganism inactivation by ohmic heating created

electroporation. The amount of exuded intracellular materials was greater with the ohmic heating compared to the conventional heating under similar time-temperature history, thereby supporting the role of electroporation. Furthermore, the morphological differences identified between the two treatments also supported the above conclusion.

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