Membrane Damage Induced by Supercritical Carbon Dioxide in *Rhodotorula mucilaginosa*

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Abstract To clarify the mechanism of microbial inactivation by supercritical carbon dioxide (SCCO₂), membrane damage of *Rhodotorula mucilaginosa* was investigated within specific pressure (10 Mpa), temperature (37 °C), and treatment time (10–70 min) ranges, including cell morphological structure, membrane permeability and fluidity. SEM and TEM observations showed morphological changes in the cell envelope and intracellular organization after SCCO₂ treatment. Increase of membrane permeability was measured as increased uptake of the trypan blue dye with microscopy, and leakage of intracellular substances such as UV-absorbing materials and ions by determining the change of protein and electrical conductivity. The SCCO₂ mediated reduction in CFU ml⁻¹ was 0.5–1 log higher at 37 °C and 10 MPa for 60 min in Rose Bengal Medium containing 4 % sodium than a similar treatment in Rose Bengal Medium. Membrane fluidity analyzed by fluorescence polarization method using 1,6-diphenyl-1,3,5-hexatriene showed that the fluorescence polarization and fluorescence anisotropy of the SCCO₂-treated cells were increased slightly and gently compared with the untreated cells. The correlation between membrane damage and death of cells under SCCO₂ was clear, and the membrane damage was a key factor induced the inactivation of cells.

Keywords Membrane damage · Supercritical carbon dioxide · *Rhodotorula mucilaginosa* · Mechanism

Introduction

Supercritical carbon dioxide (SCCO₂) treatment is a fascinating alternative non-thermal sterilization technique for foods. Because pressurized CO₂ is able to inactivate vegetative micro-organisms at low temperature, it can retain the fresh-like sensory, nutritional, and physical properties of many foods by avoiding thermal effects of traditional pasteurization [1].

Knowledge of the antimicrobial action of SCCO₂ is essential to define appropriate strategies to guarantee safety and stability of SCCO₂ processed foods and to further optimize process implementation and equipment design [2]. Although researchers have been devoted to unravel the microbial inactivation mechanism of SCCO₂, there are few explanations that are well established yet and neither be generalized for all of examined microbial strains [3]. In recent years, several innovative scenarios have been proposed to reveal the antimicrobial effect induced by SCCO₂ treatment, including cell lyses due to depressurization [4], extraction of vital intracellular components, decrease of intracellular pH [5], inactivation of key enzymes [10], and cell membrane damage or modification [6]. Among these hypotheses, membrane modification/damage is considered as the most important effective factor of the inactivation mechanism of SCCO₂ treatment [2]. SCCO₂ can diffuse into the cellular membrane, then modify the membrane fluidity and increase the membrane permeability. However, there were few relative studies on cellular membrane permeability and fluidity induced by SCCO₂.

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The soil yeast *Rhodotorula mucilaginosa*, is a common environmental inhabitant and it is a widespread hazardous food-borne microorganism which can cause food spoilage, influence the quality and shorten the shelf life of food [7]. Compared with some putrefaction bacteria, the thicker cell wall, more complex membrane structure gives it more tolerance to SCCO₂.

The aim of the present study is to provide experimental data to support the hypothesis that cellular death of *Rhodotorula mucilaginosa* was due to the membrane damage resulting from SCCO₂ treatment, concerning the changes of morphological structure, membrane permeability and fluidity.

Materials and Methods

Cultivation of Microorganism

Rhodotorula mucilaginosa (API 6672073) was obtained from the key laboratory of Food Science and Technology College, Hebei Normal University of Science and Technology. Stock culture was maintained on slants of rose Bengal medium (RBM, Beijing Aoboxing Biological Technology Co. Ltd, Beijing, China) at 4 °C during our investigation. The strain was cultivated in 100 ml of Malt Extract Medium (MEM, Beijing Aoboxing Biological Technology Co. Ltd, Beijing, China) in shaking flask culture (180 r/min, 28 °C, 10 h), and then the vegetative cells were obtained in the terminal exponential growth phase.

Ten milliliters of the culture was transferred into sterile plastic tubes (50 ml) and centrifuged (4 °C, 5,000 rpm, 10 min). The harvested cells were suspended in 20 ml of 0.85 % sterile NaCl solution, washed twice, and then resuspended in the same solution for SCCO₂ treatment. The cell concentration in the resulting suspensions was approximately 1×10^6 to 1×10^7 CFU/ml.

SCCO₂ Treatment

The experimental apparatus, designed by the China Agricultural University (Beijing, China), is shown in Fig. 1. Cell suspensions were treated by SCCO₂ in static mode with the pressure of 10 MPa at 37 °C, for different treatment times (10–70 min). After each run, the whole system was sanitized with Cold Sterilant® (Minntech Corp., Minneapolis, MN, USA) according to the manufacturer's procedure. When the selected temperature was shown on the temperature controller and equilibrated, the tubes with cell suspension was placed into the vessel immediately. The vessel and decompression valve were closed and the CO₂ inlet valve was opened. Commercially available liquid CO₂ (purity of 95 %) was pumped into vessel to reach the

required pressure. After the cell suspension was exposed to CO₂ for the designated time, the pressure relief valve was opened and the pressure was released. The depressurization took approximately 2 min. The cell suspension was aseptically removed from the vessel immediately. Triplicate 1 ml samples were taken in for initial cell counting and the rest was centrifuged (4 °C, 10,000 rpm, 10 min) for subsequent analyses.

Determination of Viable Cells

The initial and surviving cell counts were obtained by duplicate spreading 0.1 ml of serially-diluted or non-diluted samples on RBM and RBM containing 4 % sodium chloride (RBMS) which was used to investigate the injured cells. Then the plates were incubated at 28 °C for 72 h. Colonies were counted in triplicate. The microbial survival rate was expressed as the logarithmic viability reduction by $\log_{10}N/N_0$ with N and N₀ representing the colony counts before and after SCCO₂ treatment, respectively.

Determination of Cell Lyses

In this study, the number of cells was counted with the hemacytometer method by the optical microscopic observation. 10 µl of the homogeneous cell suspension was injected into the counting chamber respectively, and then the cells without lyses were observed by optical microscope for counting.

Electron Microscopic Analyses of Cells

The cell suspensions, either untreated or treated (10 MPa, 37 °C, 70 min), were centrifuged at 5,000 rpm for 10 min (4 °C). The collected cells were pre-fixed with 2.5 % (v/v) glutaraldehyde solution in 0.1 mol/l phosphate buffer (pH 7.2) for at least 2 h, rinsed three times with 0.1 mol/l phosphate buffer (pH 7.2), post-fixed with 1 % OsO₄ in 0.1 mol/l phosphate buffer (pH 7.2) for 2 h, and rinsed three times with 0.1 mol/l phosphate buffer (pH 7.2). For scanning electron microscope (SME) analysis, samples were dehydrated in an aqueous ethanol solution series (30, 50, 70, 85, and 95 %, and two times at 100 % (v/v)), treated twice for 20 min each with isoamyl acetate, air-dried and coated with gold–palladium. Observation and photomicrographs were carried out with a scanning electron microscope (SEM Quanta200, FEI, the Netherlands) and operated at a voltage of 15 kV.

Transmission electron microscopy (TEM) analysis was conducted after the dehydrated samples were embedded in epon, polymerized at 60 °C for 48 h, and sectioned by Ultramicrotome (LKB 2088). The ultra-thin sections were

Fig. 1 Schematic diagram of supercritical CO₂ sterilization apparatus. The apparatus includes: 1 CO₂ cylinder, 2 CO₂ filter, 3 pressure gauge, 4 cooling unit, 5 plunger pump, 6 pressure transducer, 7 biohazard containment unit for aseptic operation, 8 high pressure vessel, 9 thermocouples, 10 thermostatic bath, 11 vacuum pump, 12 displaying panel

stained with uranyl acetate and lead citrate, and then observed with TEM (JEM-1400, JEDL, Japan) a voltage of 80 kV.

Measurement of UV-Absorbing Substances

Three milliliters of cell suspensions were centrifuged at 5,000 rpm for 10 min (4 °C). The absorbance of the supernatant was measured at both OD_{260 nm} and OD_{280 nm} with a UV-Vis spectrophotometer (UV759S, Shangfen, Shanghai, China).

Measurement of Protein and Electrical Conductivity

The protein concentration was determined by Lowry method [8].

Trypan Blue Staining

A 100 µl Trypan blue (Beijing Solarbio Bioscience & Technology Co., Ltd, Beijing, China) solution (0.4 %) was mixed with 100 µl of cell suspensions. This procedure was performed immediately after the SCCO₂ treatment. After staining for 10 min, appropriate volume mixture was transferred immediately onto the microslide fixed coverslip and observed by microscope.

Analysis of Cells Membrane Fluidity

Fluorescence polarization method was used in this study. 1,6-diphenyl-1,3,5-hexatriene (DPH) stock solution was prepared as follows: 0.464 mg DPH (Fanbo Biochemicals Co. Ltd,

Beijing, China) was dissolved in 1 ml of tetrahydrofuran (THF) and stored in the dark at 4 °C. Before using, the stock solution was diluted with PBS (0.02 mol/l, pH 6.8).

The cell suspensions (1 ml) were centrifuged at 5,000 rpm for 10 min (4 °C), the sedimentation were washed once by the sterile 0.85 % NaCl solution, resuspended in the same solution, added to 4 µl of the diluted DPH solution, and then incubated at 30 °C for 60 min in the dark. The solutions were centrifuged at 5,000 rpm for 10 min (4 °C). The pellets were rinsed twice by the sterile 0.85 % NaCl solution at the same condition and then resuspended. Untreated samples were taken as control. The samples labeled by DPH was used to fluorescence polarization

Fig. 2 Amount of *Rhodotorula mucilaginosa* cells observed by the microscope during SCCO₂ treatment at 10 MPa and 37 °C. N: the number of visible cells observed by the microscope. The hemacytometer method was used to count

Fig. 3 SEM (a–d) and TEM (e–f) micrographs of *Rhodotorula mucilaginosa*. Image **a** (10 μm), **c** (2 μm), **e** represents untreated cells, while image **b** (10 μm), **d** (2 μm), **f** shows cells treated with SCCO₂ at 10 MPa and 37 °C for 70 min

analysis by a spectrofluorometer (F-7000, Hitachi, Japan) equipped with polarizer. The excitation and emission wavelength were observed at 358 and 429 nm respectively.

Statistical Analysis

Analyses of variance (ANOVA) were carried out by using the software Microcal Origin 7.5 (Microcal Software, Inc., Northampton, USA). The ANOVA test was performed for all experimental runs to determine significance at 95 % confidence interval. All experiments were performed in triplicates.

Results and Discussion

Cell Deformation of the Flash Depressurization

There is a hypothesis that the flash decompression will induce the mechanical cellular lyses after SCCO₂ treatment [9]. The microscopic observation showed that the quantities of the visible cells in the SCCO₂-treated and untreated groups were not different significantly and the treated cells maintained the integrity of the cell contour (Fig. 2). This indicated that the mechanical cell bursting did not happen and the effect of explosive depressurization may be much

Fig. 4 Comparisons of the inactivation of *Rhodotorula mucilaginosa* on Rose Bengal Medium (RBM) and Rose Bengal Medium containing 4 % sodium chloride (RBMS) at 10 MPa and 37 °C. N_0 and N represent the colony counts before and after SCCO₂ treatment, respectively

Fig. 5 Changes in the UV-absorbance at 260 (for nucleic acid) and 280 nm (for protein) of *Rhodotorula mucilaginosa* cell supernatant with SCCO₂ treatment at 10 MPa and 37 °C

lower than expected during the flash pressure release. The explosive depressurization of CO₂ would influence the survival ratio of the yeast cells to a lesser extent. The result was in agreement with the previous report [10], in which it was suggested that the microbial cells may not be lysed by explosive depressurization. However, Liu et al. [4] suggested that the decrease of cell viability was correlated with mechanical lyses of the cells, although no direct evidence was presented. These different results were probably attributed to the different structure of microorganism species, treatment conditions, or gas release rate. After all, the cell wall of *Rhodotorula mucilaginosa* was thicker than that of the other bacteria.

Fig. 6 Amount of protein leaked into the cells suspensions during the SCCO₂ treatment at 10 MPa and 37 °C. The absorbance was measured at 595 nm with spectrophotometer. The leakage of protein was obtained from the standard curve

Fig. 7 Electrical conductivity of supernatant collected from cell suspensions during SCCO₂ treatment at 10 MPa and 37 °C

Observation of Cell Structure Using Electron Microscopy

SEM and TEM were used to reveal the morphological structure differences between the treated and untreated cells (Fig. 3). The SEM images did not show any mechanical lyses or holes in the treated yeast cells. But their envelope deformed and wrinkled apparently compared to the untreated cells. Probably the cellular lipid extraction, a characteristic of SCCO₂, led to the collapse of cell wall, so that the cellular shape changed after the treatment [11]. Likewise, some obvious changes of the SCCO₂-treated cells were observed by TEM compared with the untreated cells. Dispersal of cytoplasm was asymmetrical; empty areas appeared in the cytoplasm, density of cytoplasm reduced, and intercellular materials gathered erratically. These phenomena were attributed to the properties of SCCO₂, which was lipophilic, and easy to diffuse into the lipid bilayer with a low viscosity and high diffusivity, and then disrupted the cell cytoplasm [12, 13].

It suggested that the cell membrane may have been damaged enough to release cellular materials to the external environment due to the loss of normal barrier function caused by SCCO₂.

Cell Injury and Death

In this experiment, different survival rates of the yeast cells due to different injury and death rate were compared in RBM and RBMS. The cells with injured membrane can not adapt to environment with a high osmotic pressure due to high salt concentration, so that they can not survive in the RBMS. Viability of the SCCO₂-treated cells in RBM and RBMS were compared (Fig. 4). The viable counts of SCCO₂-treated cells in RBMS did not decrease obviously within 30 min compared with the cells in RBM. But after 30 min, SCCO₂-treated cells showed more reduction in RBMS by about 0.5–1 log than that of cells in RBM. It was possible that when the processing time was less than 30 min, the cells membrane was affected slightly. The minor injury in cells could be repaired by its self-regulating function to maintain the membrane integrity and keep the normal physiological activities. Hong and Pyun [6] reported that *L. plantarum* cells were injured distinctly at the initial stage, which is slightly different from the results in the present study. Probably, this different result was attributed to the different species of microorganism. The membrane of *Rhodotorula mucilaginosa* was thicker and more complex than other prokaryotes so that it has more tolerance to the SCCO₂.

Permeability of Cell Membrane

Membrane permeability of *Rhodotorula mucilaginosa* as a function of the treatment time is illustrated in Fig. 5. Furthermore, the amount of leaking protein that was calculated according to the calibration curve showed the similar tendency as UV-absorbing substances (Fig. 6).

As we all know, electrical conductivity can directly reflect the concentration of ions. In this study, the electrical conductivity of supernatant was measured to demonstrate the leakage of ions out of the cells (Fig. 7). The electrical conductivity of supernatant, which was from the SCCO₂-treated cell suspension, increased gently and slightly within 60 min, but steeply after 60 min. The increase of electrical conductivity mainly resulted from the leakage of some positive ions (Ca²⁺, Mg²⁺, Na⁺, K⁺), which played important roles in contributing to the electrical conductivity. This result agreed with Hong and Pyun [6], who reported that Mg²⁺ and K⁺ ions leaked into extracellular environment after SCCO₂ treatment. It suggested that the steep increase in electrical conductivity indicated the severe damage of cell membrane.

Staining on the Cells

Trypan blue exclusion test was used to determine the integrity of cell membrane. Results showed that, the untreated cells kept their original color, but some SCCO₂-treated cells took up the dyes and turned blue (Fig. 8). The observation indicated that the membrane of some SCCO₂-treated cells was damaged and lost their integrity immediately. The longer treatment time, the more cells were injured. However, the amount of the stained cells was remarkably less than the previous report [6].

Fluidity of Cell Membrane

Fluidity change is commonly considered to be another indicator for damage of cell membrane. In this experiment, the fluidity of membrane was assessed by the fluorescence polarization and fluorescence anisotropy of the cells exposed to SCCO₂. As shown in Fig. 9, The fluorescence polarization and fluorescence anisotropy of the SCCO₂-treated cells were increased slightly compare with the untreated cells. This suggested that the fluidity of the cell membrane was decreased

Fig. 8 Trypan blue staining of *Rhodotorula mucilaginosa* untreated (a), SCCO₂-treated for 20 min (b), and SCCO₂-treated for 60 min (c) at 10 MPa and 37 °C

Fig. 9 Amount of the fluorescence polarization and fluorescence anisotropy of cellular membrane of *Rhodotorula mucilaginosa* during SCCO₂ treatment at 10 MPa and 37 °C

after SCCO₂ treatment [14]. It was reported that the fluorescence polarization were inversely proportional to membrane fluidity. The depression of membrane fluidity induced by SCCO₂ was demonstrated in a model cellular membrane, which was aqueous dipalmitoylphosphatidylcholine (DPPC) liposomes, and in a thermophilic bacterium model (*Clostridium thermocellum*) [15]. Their results showed that the decline of membrane fluidity also occurred in a lower extent, the same as our finding.

Conclusions

We can conclude that the cell membrane damage is a key factor induced the inactivation of *Rhodotorula mucilaginosa* cells by SCCO₂. SCCO₂ treatment increases the membrane permeability and decreases the membrane fluidity.

However, further investigations are required to precisely elucidate why the high-pressure CO₂ treatment had no burst effect on the cell wall and what mechanism dominates under various process conditions.

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