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Comparison of a two-stage system with low pressure carbon dioxide microbubbles and heat treatment on the inactivation of *Saccharomyces pastorianus* cells

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ABSTRACT

To clarify how *Saccharomyces pastorianus* cells were affected by a two-stage system that was heating and pressurizing after microbubbled carbon dioxide (MB-CO_2) was mixed with the *S. pastorianus* suspension at low temperature and pressure (two-stage MB-CO_2), *S. pastorianus* cells were observed by electronic microscopy and stained with propidium iodide (PI). Furthermore, the amounts of nucleic acid and protein leaked from treated *S. pastorianus* cells were determined and intracellular enzyme activities were measured. It was observed by scanning electric microscopy that wrinkles in *S. pastorianus* cells treated by two-stage MB-CO_2 with a heating coil at 50 °C (MB50) and heat treatments at 50 °C and 80 °C (H50 and H80) were more than those in untreated (NT) cells. Upon observation with transmission electron microscopy, it suggested that MB50 had a direct effect on the intracellular substrate, although little influence on the membrane, whereas H80 cells showed visible damage to cell membranes. However, it was recognized that PI intensity in MB50 cells was great than that in NT, H50 and H80 cells, and that the amount of nucleic acid and protein leaked from H80 cells was significantly higher than that of NT, MB50 and H50 cells. Furthermore, the enzyme inactivation efficiency in MB50 cells was the same as in H80 cells. These results estimate that inactivation of *S. pastorianus* by two-stage MB-CO_2 was due to actions of MB-CO_2 on the cell membrane and the intracellular substrate such as enzyme inactivation.

1. Introduction

Pressurized carbon dioxide (CO_2) processes that include supercritical (SC-) CO_2 as alternatives for food pasteurization have been widely studied because heating may cause undesirable changes in the taste and flavour of foods (Damar & Balaban, 2006; Garcia-Gonzalez, Geeraerd, Spilimbergo, et al., 2007; Spilimbergo & Bertucco, 2003). However, from a practical perspective, the SC-CO_2 process is prohibitively expensive because it requires maintaining high-pressure conditions (10–30 MPa) in order to effectively inactivate microorganisms. Furthermore, SC-CO_2 may also result in a loss of food flavour because it is used as an extracting solvent for organic compounds (Chen et al., 2009; Díaz-Maroto, Pérez-Coello, & Cabezudo, 2002; Kazazi, Rezaei, Ghotb-Sharif, Emam-Djomeh, & Yamini, 2007).

We recently developed a low pressure CO_2 microbubbles (MB-CO_2) system equipped with an MB generator based on a gaseous CO_2 pressure less than SC-CO_2 that could effectively inactivate *Escherichia coli*, *Saccharomyces cerevisiae* and *Lactobacillus fructivorans* (Kobayashi et al., 2009;Kobayashi, Hayata, Ikeura, Muto, & Osajima, 2010; Kobayashi, Ikeura, Odake, Tanimoto, & Hayata, 2012; Kobayashi, Sugawara, et al., 2012). In addition, to improve the MB-CO_2 efficiency, the MB-CO_2 equipment was devised from a batch system to a two-stage system that was heating and pressurizing MB-CO_2 when mixed with a model solution at low temperature and pressure. It was then reported that the quality of sake in which *L. fructivorans* and some enzymes were inactivated by the two-stage MB-CO_2 retained good taste and flavour on sensory evaluation and measurement of volatile compounds (Kobayashi, Ikeura, Odake, & Hayata, 2013a). However, the mechanisms of action of two-stage MB-CO_2 on microbial cells are still not clear.

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Beer yeast (*Saccharomyces pastorianus*) was used in the study. Beer yeast and some bacteria present in fermented beer cause the quality to deterioration (Miyamoto, 2001), so it is treated by pasteurization at 70 °C or by filtration. However, changes in the taste and flavour of pasteurized beer are induced by heating. At some beer companies in Japan, preservation and distribution of unpasteurized beer is permitted at room temperature after removing solid materials such as substances bound to proteins and polyphenols, hop resins, yeasts and some bacteria in fermented beer using filtration (Inui, 2001). In recent years, unfiltered beer that contains yeast has become popular in Japan due to its mellow taste and rich flavour. In addition to its unique taste and flavour, this type of beer also contains some nutrients, including amino acids, vitamins and minerals (Arimura, 2000; Sogawa, 1990). However, it is not widely distributed due to its short shelf-life. Therefore, it is considered that beer containing yeast would be more widely distributed if the yeast could be inactivated without heating.

In this study, to investigate the effect of two-stage MB-CO_2 to the membranes and interiors of *S. pastorianus* cells, observation with scanning and transmission electron microscopy and fluorescence analysis with propidium iodide were performed. The amounts of protein and nucleic acid leaked from *S. pastorianus* cells were estimated by a spectrophotometer and intracellular enzyme activities were measured with the APIZYM kit.

2. Materials and methods

2.1. Preparation of S. pastorianus suspension

S. pastorianus NBRC 11024 was inoculated into a test tube containing 10 mL of YM medium (Difco, Becton Dickinson, Franklin Lakes, NJ) and incubated at 25 °C for 8 h. Next, the culture was transferred to a conical flask containing 190 mL of YM medium and incubated at 25 °C for 14 h. The cells were then collected by centrifugation (5 °C, 10,000× *g*, 30 min) and re-suspended in 10 L of physiological saline containing 5% ethanol (approximately 1.0×10^6 CFU/mL).

2.2. The two-stage MB-CO_2 apparatus and procedure for treating the model solution

The experimental equipment for the two-stage MB-CO_2 used in the present study has been previously reported (Kobayashi, Ikeura, Odake, & Hayata, 2013b).

The two-stage MB-CO_2 treatment was performed under the following conditions: the temperature and pressure in the mixing vessel were set to 5 °C and 1.0, 1.5, or 2.0 MPa, respectively; the temperature, pressure and exposure time in the heating coil were then set to 35 °C, 40 °C, 45 °C, or 50 °C, 4.0 MPa, and 1, 3, or 5 min, respectively.

2.3. Heat treatment

For comparison, heat treatment alone was performed at 35°C–80 °C using only heating coil in the two-stage MB-CO_2 apparatus.

2.4. Measurement of numbers of surviving S. pastorianus cells

The number of surviving *S. pastorianus* cells in each sample was measured by plating 1 mL each of the sample or diluted sample on duplicate YM agar plates. The plates were then incubated at 25 °C for 48 h. After incubation, plates with 30–300 CFU were chosen and the colonies counted. If there were only low numbers of viable cells, colonies on plates from the undiluted sample were counted (Kobayashi, Sugawara, et al., 2012). The detection limit was 1 CFU/mL. All experiments were performed in triplicate. The data presented are the means with standard errors of the results from triplicate experiments.

2.5. Morphological observation of S. pastorianus cells by electron microscopy

After 100 mL of *S. pastorianus* suspension was mixed with 8 mL of 25% glutalaldehyde (Kanto Chemical Co., INC., Tokyo, Japan), pre-fixed at 5 °C for 48 h and post-fixed by 2% OsO_4 solution for 1 h, it was then serially dehydrated for 20 min each in 30%, 50%, 70%, 80%, 90%, 99.5% and 100% dehydrated ethanol. Dehydration with 99.5% and 100% dehydrated ethanol was performed twice.

The observation by SEM was performed as follows: The dehydrated samples were immersed in a mixture of t-butyl alcohol and dehydrated ethanol (1:1) and allowed to remain so for a moment. The samples were then freeze-dried with a freeze drier (ES-2030, Hitachi High Technologies Co., Tokyo, Japan), OsO_4-coated with a OsO_4 coater (HPC-1SW, Vacuum device Inc., Mito, Japan) for 10 s under vacuum and afterward replaced in 100% t-butyl alcohol and frozen overnight at −20 °C (the thickness of the coating was adjusted to 3 nm). Then the samples were observed with SEM (JSM-6700F, JEOL Ltd., Akishima, Japan) operated at 3 kV.

The observation by TEM was performed as follows: The dehydrated samples were immersed in a mixture of Quetol-651 (Cosmo Bio Co., Ltd. Tokyo, Japan) and dehydrated ethanol (1:1) and allowed to remain so for a moment. Ultra-thin sections (thickness 70–100 nm) were made from samples embedded in 100% Quetol-651 at 60 °C for 48 h with an ultramicrotome (ULTRA CUT UCT, Leica Microsystems, Wetzlar, Germany). The ultra-thin sections were doubly electron-strained with uranyl acetate and lead nitrate and then observed with TEM (JEM-2010, JEOL Ltd.) operated at 200 kV.

2.6. Fluorescence analysis with propidium iodide

S. pastorianus cells were fluorescently-stained with propidium iodide (PI, Dojindo Raboratories, Kumamoto, Japan). PI is a fluorescent indicator that passes through the membranes of dead cells and intercalates with nucleic acid. A PI solution (4 μmol/L in PBS) was mixed with the *S. pastorianus* suspension (10^6 CFU/mL) at a ratio of 1:2 and kept at 37 °C for 15 min. The fluorescence intensity of the mixture was measured with a fluorescence spectrophotometer (RF-5300PC, Shimadzu Co., Kyoto, Japan) at excitation and fluorescence wavelength of 545 and 605 nm, respectively.

2.7. Measurement of nucleic acid and protein leaked from S. pastorianus cells

Measurements of nucleic acid and protein leaked from *S. pastorianus* cells were performed by measuring a supernatant filtered through a 0.22 μm filter with a spectrophotometer (UV-1700 PharmaSpec, Shimadzu Co., Kyoto, Japan) at 260 and 280 nm, respectively.

2.8. Measurement of enzyme activities in S. pastorianus cell by the APIZYM kit

Enzyme activity in *S. pastorianus* cells was measured with an APIZYM kit (BioMerieux, Marcy-l'Etoile, France). This kit permits monitoring of 20 different constitutive enzyme activities from a complex sample that has not been purified (Ballestra, Da Silva, & Cuq, 1996). The number of *S. pastorianus* cells used to measure the enzyme activity was adjusted to approximately 1.0×10^7 CFU/mL by centrifugation. All enzyme activities were visually measured