

Effect of Low-pressure Carbonation on the Heat Inactivation and Cytoplasmic Acidification of *Saccharomyces cerevisiae*

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Received: October 8, 2012 / Published: December 20, 2012.

Abstract: Effect of low-pressure carbonation (LPC) on heat inactivation of *Saccharomyces cerevisiae* was investigated. The cell suspension was carbonated at 1 MPa and 4 °C for 15 min and subsequently heated from 51 to 61 °C and 5 s to 5 min (heating with LPC). As a control experiment, cell suspension was heat-treated under atmospheric pressure without LPC (heating). The inactivation ratio of heating at 53 °C and 55 °C for 1 min with LPC was approximately 1 log order higher than heating alone. Extending heating time to 5 min did not widen the difference in the inactivation ratio between heating with LPC and heating alone at both heating temperatures. At 57 °C, the difference in inactivation ratio increased from 1 to 2.5 log order with extending treatment time from 5 to 15 s. The results suggested that the enhanced inactivation effect by LPC was obtained at the higher temperature with short time treatment than the lower temperature with longer time treatment. Under fluorescence microscope observation of LPC-treated cell stained with LysoSensor probe, it seemed that LPC was hardly able to acidify the cytoplasm of *S. cerevisiae*. It is considered that the ability of *S. cerevisiae* cells to keep their cytoplasmic pH during LPC resulted in the inferior increase in heat inactivation ratio by LPC as compared with bacteria in the previous studies.

Key words: *Saccharomyces cerevisiae*, low-pressure carbonation, heat inactivation, heat sensitivity.

1. Introduction

The incidents of microbial deterioration of liquid foods such as several kinds of fruit juice are the most frequently due to the fermentative yeast, *Saccharomyces cerevisiae* [1, 2] which causes ethanolic spoilage, production of hydrogen sulfide and other off-odors [3]. Therefore, it is necessary to eliminate *S. cerevisiae* to provide an appropriate shelf-life of the liquid foods.

Heat treatment is the most frequently accomplished in commercial pasteurization of liquid foods. Although it can provide high inactivation effect, the heat treatment with severe conditions typically produces

cooked flavors which can affect the original aromas and flavors of the fresh liquid foods. Furthermore, it can cause the loss of nutrients and vitamins. To meet the consumer's requirement for fresh-like liquid foods, there is a growing interest in non-thermal processes for the preservation. In addition, preservatives such as benzoic acid can exhibit optimum activity in the pH range of 2.5-4.0 [4]. Sulfur dioxide and sorbic acid are also commonly used to inhibit mold and yeast in fruit juices [5]. Besides, the application of irradiation technology is available and now legally permitted in more than 40 countries such as USA, Germany, France and Brazil [6]. Unfortunately, consumer attitudes toward the use of the preservatives and irradiation have so far limited. Removal of microorganisms by physical methods such as centrifugation and ultrafiltration are also reported. Other physical methods, such as

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utilization of pulsed electric fields and high hydrostatic pressure, are also widely studied [7]. High hydrostatic pressure has been investigated as a method to stabilize liquid foods at reduced temperatures by inactivating the microbial cells [8]. The high-pressure treatment of 400 MPa at 20 °C for 30 min was revealed to cause a 3.51 log reduction of *S. cerevisiae* [9]. Nevertheless, the large financial investment required for the high pressures equipment [10].

CO₂ is accepted as an innocuous ingredient in foods because it is nontoxic, inflammable, inexpensive and environmentally safe. Water-dissolved CO₂ also inactivates various microbial cells [11, 12]. A 7 log order of *S. cerevisiae* cells can be inactivated at 1,000 psi (6.9 MPa), for 15 min at room temperature [10]. Although there are many studies which investigated the effect of high-pressure and supercritical CO₂ in microbial inactivation, the effect of the low-pressure is quite rarely studied. In previous study, we investigated that low-pressure carbonation (LPC) can not significantly inactivate microbial cells itself, but can increase the heat sensitivity of the microbial cells. We carbonated several microbial cell suspensions and subsequently heated for 1 min at temperatures ranging from 50 °C to 70 °C at 1 MPa compared with heating under atmospheric pressure, heating with LPC exhibited 3-5 log inactivation on several bacteria [13]. In present study, we decided to explore the most appropriate condition of heating temperature and time under the LPC in an effort to facilitate the LPC's enhancing effect on heat inactivation of *S. cerevisiae*.

2. Materials and Methods

2.1 Preparation of Cell Suspension

S. cerevisiae NBRC 10217 was obtained from the National Institute of Technology and Evaluation (Tokyo, Japan). The cells were precultured in 5 mL of yeast malt peptone (YM) broth (Difco, Detroit, USA) at 30 °C for 24 h, and 3 mL of the resulting culture was inoculated into 200 mL of freshly prepared YM broth. This culture was incubated to attain the late logarithmic

growth phase. Cells were harvested and washed three times by centrifugation at $2,000 \times g$ at 4 °C for 10 min in 0.85% w/v sodium chloride solution, and the pellet was resuspended in the solution to yield a final cell concentration of 10^8 colony forming units (CFU)/mL. This cell suspension was subjected to the inactivation treatments described below.

2.2 Inactivation Treatments

Three treatments (LPC, heating with LPC and heating) were performed. Schematic diagram of the apparatus used for the LPC and heating is shown in Fig. 1.

2.2.1 LPC

The *S. cerevisiae* cell suspension was poured into a CO₂-dissolving vessel. CO₂ gas was then introduced into the vessel at a pressure of 1 MPa, and dissolved into the cell suspension by stirring at 4 °C for 15 min to the point of saturation. The saturation of CO₂ in the cell suspension was confirmed by the method of Shimoda [14].

2.2.2 Heating with LPC

The LPC-treated suspension was introduced into the residence column adjusted to 51-61 °C in a water bath. After heating for 5 s-5 min in the residence column at 1 MPa, the cell suspension was withdrawn through a flow rate control valve. During heating, the CO₂ concentration in the cell suspension was kept saturated at 1 MPa at each temperature.

2.2.3 Heating

The *S. cerevisiae* cell suspension in the CO₂-dissolving vessel was introduced into a residence column in the water bath using the pressure of oxygen gas. After heating at 51-61 °C for 5 s-5 min in the column under atmospheric pressure (0.1 MPa), the cell suspension was withdrawn through a flow rate control valve.

2.3 Determination of Inactivation Ratio

Appropriate serial dilutions of each sample were prepared in a 0.85% sodium chloride solution; 0.1 mL of the suspension was plated onto YM agar (Difco,

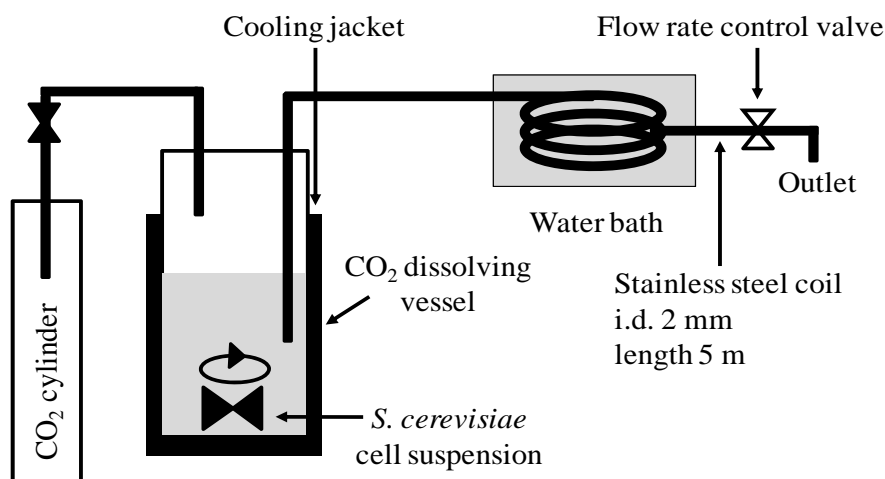


Fig. 1 Schematic diagram of the apparatus used for LPC and heating.

Detroit, USA) plate; and the number of forming colony was enumerated after incubation at 30 °C for 48 h. The inactivation ratio was expressed as $\log(N_0/N)$, where N_0 is the initial colony count and N is the colony count after each treatment. The inactivation ratio was determined as the mean \pm standard deviation of three independent experiments.

2.4 LysoSensor Probe Staining

To evaluate the effect of LPC on cytoplasmic acidification of *S. cerevisiae* cells, the LPC-treated (1 MPa, 4 °C, 15 min) *S. cerevisiae* cells were stained with LysoSensor Yellow/Blue DND-160 (Molecular probes, Invitrogen Inc., OR, USA), according to the manufacture's protocol, the fluorescent pH indicator partitioning into acidic organelles. The LysoSensor-stained cells were observed under a microscope (BX 50, Olympus Co.) equipped with accessories for fluorescence observation (BH2-RFL-T3, U-ULS100HG, and BX-KLA; Olympus Co.). In addition, the high pressure carbonation (HPC)-treated cell (5 MPa, 30 °C, 15 min) was also observed as a positive control for the acidified cell.

3. Results and Discussion

3.1 Effect of Heating with LPC on the Inactivation of *S. cerevisiae* Cells

Inactivation effects of 51 °C to 61 °C heating

temperature to *S. cerevisiae* were compared between heating and heating with LPC for 1 min heating time (Fig. 2). At 51 °C, the inactivation ratio of heating was almost not able to be detected and the same result was also observed when heating with LPC. Heating at 53 °C and 55 °C with LPC showed higher inactivation ratio than heating alone for 1.15 and 1.25 log order, respectively. In addition to the previous study, herein it can be firstly suggested that not only the bacteria [13], but also *S. cerevisiae* LPC can increase the heat sensitivity. However, the LPC-increased heat sensitivity of *S. cerevisiae* seemed to be different depending on the level of heating temperatures. At the heating temperature of 57 °C, the enhanced heat inactivation level by LPC was smaller than the inactivation level at 53 and 55 °C, and gradually decreased with the increasing temperature. It was proposed that at a particular heating temperature and time, the acquisition of heat resistance of *S. cerevisiae* can be triggered [15]. From this, it can be suggested that the heat sensitivity of *S. cerevisiae* decreased during the heating temperatures rising from 59 to 61 °C at 1 min heating time. Besides, the LPC-increased heat sensitivity also decreased during the temperatures rising from 57 to 61 °C.

Fig. 3 shows the effect of the heating time from 1 to 5 min on the inactivation ratio of *S. cerevisiae* at 53 °C (Fig. 3a) and 55 °C (Fig. 3b) when heating with LPC

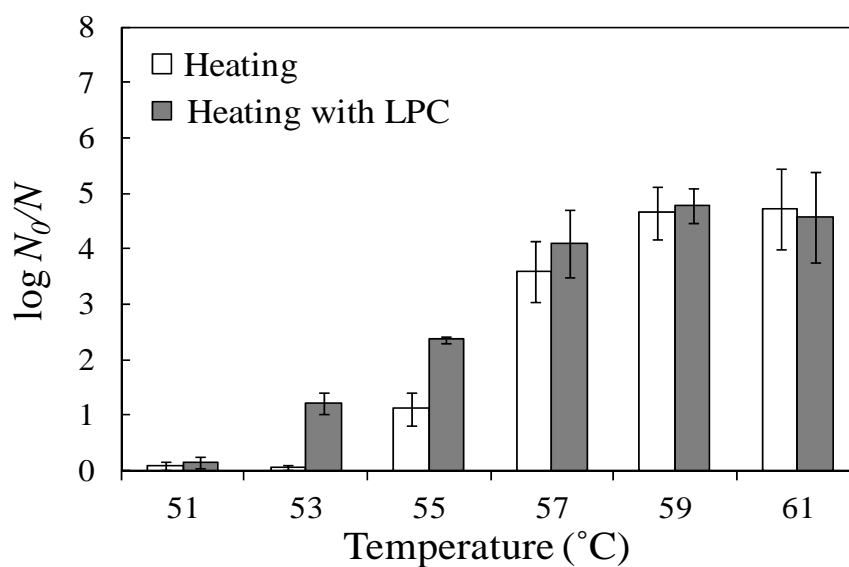


Fig. 2 Effect of heating (0.1 MPa, 51-61 °C, 1 min) and heating with LPC (1 MPa, 51-61 °C, 1 min) on log inactivation ratio of *S. cerevisiae*. The initial cell concentration was 10^8 CFU/mL.

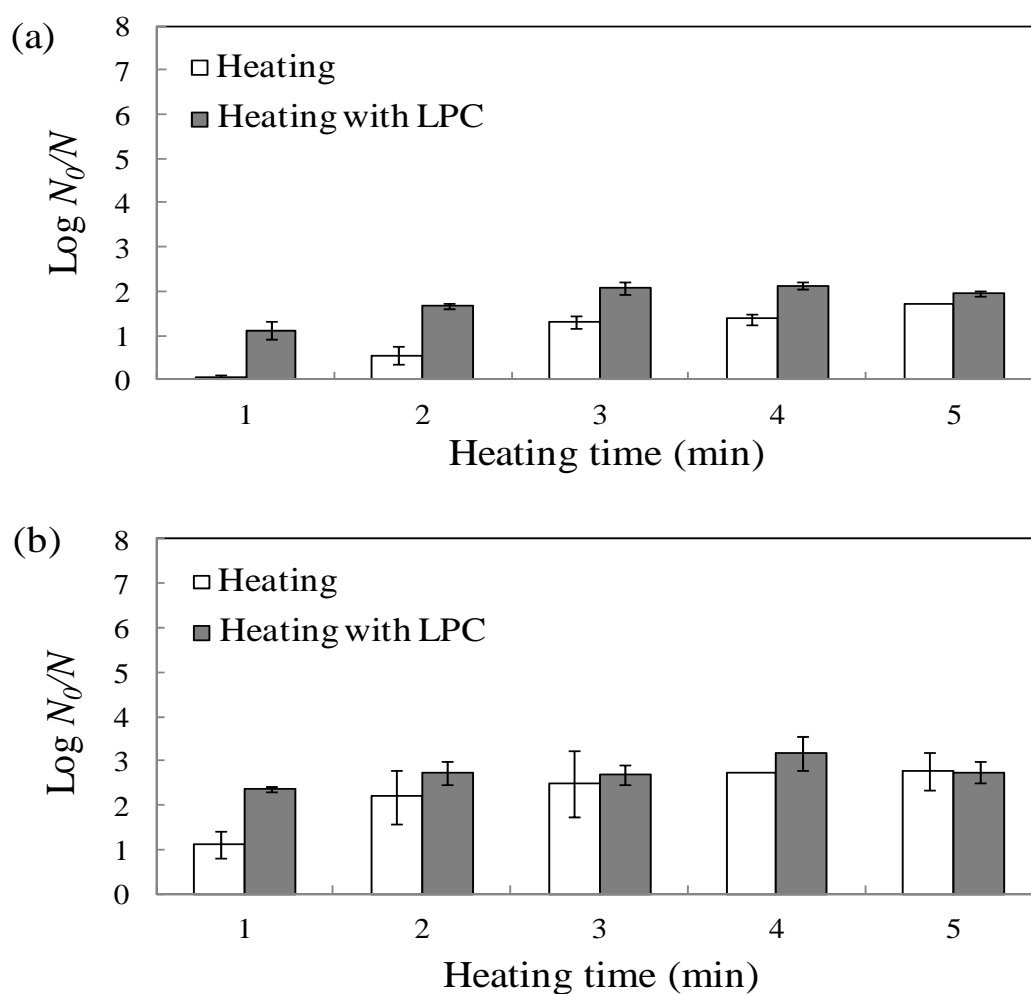


Fig. 3 Effect of heating treatment time on log inactivation ratio of *S. cerevisiae* after heating (0.1 MPa, 1 min) and heating with LPC (1 MPa, 1 min). (a) 53 °C; (b) 55 °C. The initial cell concentration was 10^8 CFU/mL.

and heating alone. At 53 °C for 1 and 2 min treatment, LPC could enhance the heat inactivation for 1.15 and 1.14 log order, respectively. Then, the enhanced inactivation ratio gradually decreased with the extending heating time to nearly undetectable at 5 min heating time. At 55 °C for 1 min treatment, LPC could enhance the heat inactivation for 1.25 log order. Then, the enhanced inactivation ratio decreased to 0.54 log orders at 2 min treatment and gradually decreased with the extending heating time. Therefore, at 53 °C and 55 °C, the enhanced heat inactivation level by LPC could be decreased even extending the heating time.

Fig. 4 shows the inactivation ratio during the shorter treatment time from 1 to 15 s. Temperature was set to 57 °C because *S. cerevisiae* cells were effectively inactivated at 57 °C (Fig. 2). The results showed that the LPC-enhanced inactivation ratio increased from 1 to 2.5 log orders with the extending treatment time from 5 to 15 s. From these results, it can be concluded that when performing specific short heat treatment period, the LPC-enhanced inactivation level increased with the extending heating time.

3.2 Effect of LPC on the Cytoplasmic Acidification of *S. cerevisiae* Cells

We previously revealed that LPC can enhance the

heat inactivating effect on bacteria to 3-5 log orders at several heating temperatures [13]. However, the increased inactivation effect enhanced by LPC was smaller in *S. cerevisiae* compared to bacteria. The accomplishment to enhance heat inactivation of LPC on bacterial cells was concluded in our previous study. We have found that the increased inactivation ratio was represented by the rise in cytoplasmic proton concentration in bacterial cell during LPC which led to the acidification of the cell. LPC can decrease the intracellular pH of *E. coli* to 5.5 when the inactivation ratio was enhanced from heating alone for 3 log orders [16]. This phenomenon can cause many physiological damages within the cell, resulting in the decreasing heat resistance [16, 17]. It was proposed that *S. cerevisiae* cells are less sensitive to CO₂ concentration than bacterial cells because they have vacuoles which can prevent the cytoplasmic acidification of the cell by pumping out an excess proton via V⁺-ATPase [18]. Therefore, we evaluated cytoplasmic pH level of *S. cerevisiae* cells after LPC by staining the cell with Yellow/Blue DND-160, and used the fluorescent acidotropic LysoSensor probe for labeling and tracking acidic organelle. In acidic organelles Yellow/Blue DND-160 has predominantly yellow fluorescence, and in less acidic organelles it has blue fluorescence. The

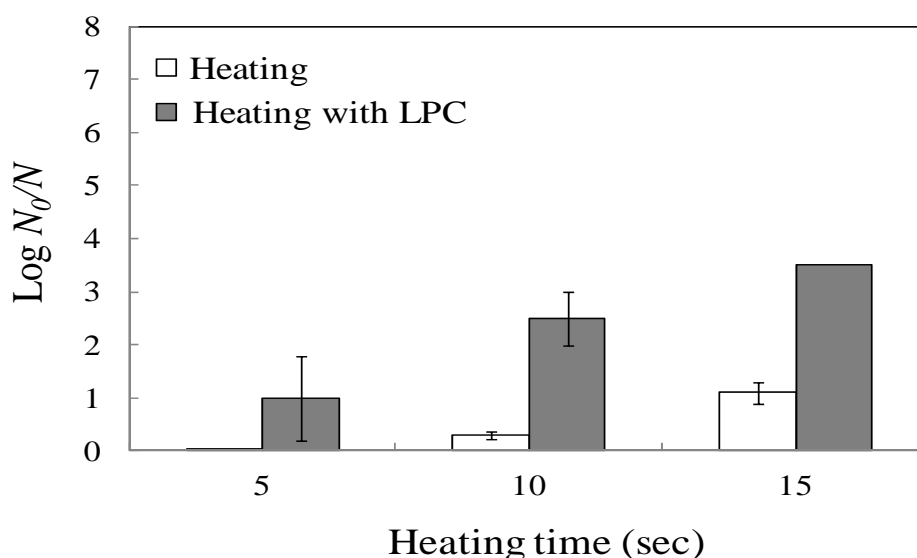


Fig. 4 Effect of heating treatment time on log inactivation ratio of *S. cerevisiae* after heating (0.1 MPa, 57 °C, 5-15 s) and heating with LPC (1 MPa, 57 °C, 5-15 s). The initial cell concentration was 10⁸ CFU/mL.

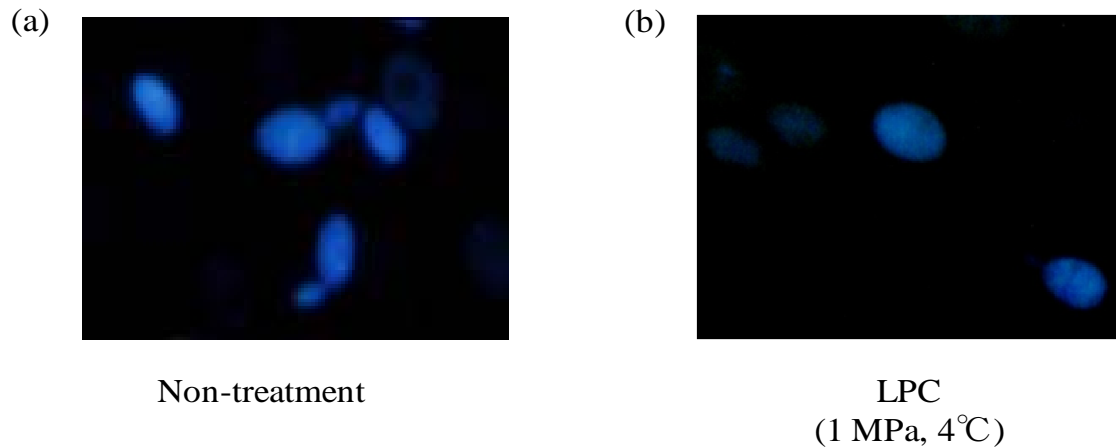


Fig. 5 LysoSensor staining properties of *S. cerevisiae* cells. (a) non-treatment, (b) LPC under 1 MPa, 1 min.

results showed that similar to the untreated cell (Fig. 5a), the LPC-treated cell under 1 MPa, 4 °C, 15 min showed blue fluorescence (Fig. 5b). Hence, it can be implied that LPC is macroscopically unable to acidify the cytoplasm of *S. cerevisiae* cell, resulting in the inferior inactivation ratio compared with bacterial cell. However, with short time heat inactivation, the effect of LPC to enhance the inactivation of *S. cerevisiae* was observed. Additional research is necessary to further explain the specific effect.

4. Conclusions

Short time (5 to 15 s) with higher temperature (57 °C) is the promised condition for heating with LPC that can effectively inactivate *S. cerevisiae*. The practical application of the explored LPC condition could be proposed as an alternative technique for inactivation of *S. cerevisiae* in various liquid foods. As the heating condition can be operated for short time, the extent of degradation of the food flavors and nutrition can be reduced.

References

- [1] M.E. Parish, D.P. Higgins, Yeasts and molds isolated from spoiling citrus products and by-products, *Journal of Food Protection* 52 (1989) 261-263.
- [2] M.E. Parish, Microbiological concerns in citrus juice processing, *Food Technology* 45 (1991) 128-136.
- [3] C.D. Zook, M.E. Parish, R.J. Braddock, M.O. Balaban, High pressure inactivation kinetics of *Saccharomyces cerevisiae* ascospores in orange and apple juices, *Journal of Food Science* 64 (1999) 533-535.
- [4] C.R. Fellers, Sodium benzoate and benzoic acid preservatives for cider and other fruit juices, *Fruit Products Journal and American Vinegar Industry* 9 (1929) 113-115.
- [5] A.D. Warth, Resistance of yeast species to benzoic and sorbic acids and to sulfur dioxide, *Journal of Food Protection* 48 (1985) 564-569.
- [6] P. Loaharanu, International trade in irradiated foods: Regional status and outlook, *Food Technology* 43 (1989) 77-80.
- [7] B.L. Qin, F.J. Chang, G.V. Barbosa-Canovas, B.G. Swanson, Nonthermal inactivation of *Saccharomyces cerevisiae* in apple juice using pulsed electric fields, *Lebensmittel Wissenschaft und Technologie* 28 (1995) 564-568.
- [8] H. Ogawa, K. Fukuhisa, Y. Kubo, H. Fukumoto, Pressure inactivation of yeasts, molds, and pectinesterase in satsuma mandarin juice: Effects of juice concentration, pH, and organic acids, and comparison with heat sanitation, *Agricultural and Biological Chemistry* 54 (1990) 1219-1225.
- [9] F. Moerman, High hydrostatic pressure inactivation of vegetative microorganisms, aerobic and anaerobic spores in pork Marengo, a low acidic particulate food product, *Meat Science* 69 (2005) 225-232.
- [10] H.M. Lin, Z. Yang, L.F. Chen, Inactivation of *Saccharomyces cerevisiae* by supercritical and subcritical carbon dioxide, *Biotechnology Progress* 8 (1992) 458-461.
- [11] J.A. Daniels, R. Krishnamurthi, S.S.H. Rizvi, A review of effects of carbon dioxide on microbial growth and food quality, *Journal of Food Protection* 48 (1985) 532-537.
- [12] G.J. Haas, H.E. Jr. Prescott, E. Dudley, R. Dik, C. Hintlian, L. Keane, Inactivation of microorganisms by carbon dioxide under pressure, *Journal of Food Safety* 9 (1989) 253-265.
- [13] S. Noma, W. Klangpetch, S. Nakamura, T. Ishibashi, H.

- Huang, N. Igura, et al., Effect of low-pressure carbonation on heat inactivation of yeast and bacterial vegetative cells, *Food Science and Technology Research* 16 (2010) 389-394.
- [14] M. Shimoda, H. Kago, N. Kojima, M. Miyake, Y. Osajima, I. Hayakawa, Accelerated death kinetics of *Aspergillus niger* spores under high-pressure carbonation, *Applied and Environmental Microbiology* 68 (2002) 4162-4167.
- [15] P. Gervais, I. Martínez de Marañón, Effect of the kinetics of temperature variation on *Saccharomyces cerevisiae* viability and permeability, *Biochimica et Biophysica Acta* 1235 (1995) 52-56.
- [16] W. Klangpetch, S. Noma, N. Igura, M. Shimoda, The effect of low-pressure carbonation on the heat inactivation of *Escherichia coli*, *Bioscience, Biotechnology, and Biochemistry* 75 (2011) 1945-1950.
- [17] S.I. Hong, Y.R. Pyun, Membrane damage and enzyme inactivation of *Lactobacillus plantarum* by high pressure CO₂ treatment, *International Journal of Food Microbiology* 63 (2001) 19-28.
- [18] T. Watanabe, S. Furukawa, K. Kitamoto, A. Takatsuki, R. Hirata, H. Ogihara, et al., Vacuolar H⁺-ATPase and plasma membrane H⁺-ATPase contribute to the tolerance against high-pressure carbon dioxide treatment in *Saccharomyces cerevisiae*, *International Journal of Food Microbiology* 105 (2005) 131-137.