Temperature-dependency on the inactivation of *Saccharomyces pastorianus* by low-pressure carbon dioxide microbubbles

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Abstract Temperature-dependency on cell membrane injury and inactivation of *Saccharomyces pastorianus* by low-pressure carbon dioxide microbubbles (MBCO₂) was investigated. The number of surviving *S. pastorianus* cells after MBCO₂ treatment detected with yeast and mould agar (YMA, an optimum agar) was higher than that with YMA adding 2.5 g/L sodium chloride and yeast nitrogen base agar (a minimum agar). However, the decrease of the surviving number by thermal treatment was not changed among above agars used. The fluorescence polarization (FP), which indicated the phase transition of the membrane of *S. pastorianus* cells treated with MBCO₂ increased with increasing temperature. The activity of the alkaline phosphatase (AP), a periplasmic enzyme, in *S. pastorianus* cells after MBCO₂ and thermal treatments increased with the FP but was reduced by further increasing temperature. The FP and AP activities after MBCO₂ treatment increased at a temperature lower than the temperature of the thermal treatment. In addition, intracellular pH of *S. pastorianus* decreased by the MBCO₂ treatment at lower temperature with increasing pressure. Therefore, it was revealed that phase transition of the cell membrane and inactivation of *S. pastorianus* was caused by MBCO₂ treatment at lower temperature than thermal treatment and that the effect was induced by the dissolved CO₂ and increased with increasing pressure.

Keywords Cell membrane injury · Inactivation · Intracellular pH · Low-pressure carbon dioxide microbubbles · *Saccharomyces pastorianus*

Introduction

Several non-thermal sterilization techniques such as Barba et al. (2017), Guimarães et al. (2018) have been reported until now. Pressurized carbon dioxide (CO₂) is one of non-thermal sterilization techniques and has been widely studied (Amaral et al. 2017; Garcia-Gonzalez et al. 2007; Silva et al. 2018). Silva et al. (2018) evaluated the influence of CO₂ volume ratio on the inactivation of *Lactobacillus casei* by pressurized CO₂ using a two-level plackett–burman design and a box–behnen design. The mechanism on the microbial inactivation by pressurized CO₂ has not been completely understood yet. However, Howladar et al. (2017) indicated that pressurized CO₂ triggered cell disruption or cell lysing of *Rhodotorula glutinis*. Liao et al. (2010) discussed that the rigidity increase and the fluidity decrease of the cell membrane were caused by the dissolution of CO₂ of the lipid bilayer of the cell membrane as a result of pressurized CO₂ treatment. Li et al. (2012) observed morphological changes of *Saccharomyces cerevisiae* cells treated with pressurized CO₂ by electron microscopy and confirmed the fluidity decrease and the permeability increase of the cell membrane by fluorescence analysis. Tamburini et al. (2014) has proposed that the change of the cell membrane permeability is the first event on the inactivation of *Escherichia coli* by pressurized CO₂. The correlation between the cell membrane permeability and inactivation of *S. cerevisiae* and *R. mucilaginosa* by pressurized CO₂ has been furthermore reported (Li et al. 2013; Spilimbergo et al. 2010a). On the other hand, it was

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estimated that a key factor in microbial inactivation by pressurized CO₂ was the lowering of intracellular pH (pH_{in}) (Spilimbergo et al. 2010b). Intracellular acidification of *E. coli* and *S. cerevisiae* by pressurized CO₂ has been recognized (Watanabe et al. 2005). Giulitti et al. (2011) additionally demonstrated that both pH_{in}-lowering and membrane permeability increase were important factors on microbial inactivation by pressurized CO₂.

In recent years, extremely minute bubbles with diameters of 50 µm or less, known as microbubbles (MB), have gained attention in food science and agriculture. Compared to millibubbles that are normally generated using air pumps, MB rise more slowly, have extremely high solubility because they compress, dissolve and disappear in water (Takahashi et al. 2007a, b; Li et al. 2009a, b). Therefore, we designed an equipment in which MBCO₂ was fed into liquid samples at pressure, and reported that yeast in unfiltered beer and *hiochi* bacteria in unpasteurized sake were inactivated by the MBCO₂ (Kobayashi et al. 2014; Kobayashi and Odake 2015). In the inactivation mechanism of *S. pastorianus* by MBCO₂, it was proposed that the inactivation occurred due to the denaturation of the intracellular components by multiple factors such as the temperature increase, the pH_{in}-lowering and the action of CO₂ molecules (Kobayashi and Odake 2018). However, these actions have not been verified yet.

The following agars have been used for detecting bacteria injured by sterilization treatment; an agar adding sodium chloride to an optimum agar (live bacteria that injured cell membrane cannot grow) and a minimum agar (live bacteria that induced a lack of permselectivity and complex auxotrophy by injuring cell membrane cannot grow) (Straka and Stokes 1959; Hurst et al. 1973). Furthermore, the fluorescence polarization (FP) has been used for measuring phase transition of the cell membrane (Liao et al. 2010), and it has been reported that alkaline phosphatase (AP), a periplasmic enzyme (Nikerson et al. 1948), activates in bacterial cells with changing the membrane fluidity by thermal treatment (Katsui et al. 1982; Tsuchido et al. 1985). In this study, to clarify the temperature-dependency on the inactivation and the phase transition of cell membrane of *S. pastorianus* by MBCO₂, the incubation test with above agars, and the measurements of FP, AP activity and pH_{in} were performed.

Materials and methods

Preparation of *Saccharomyces pastorianus* suspension

As with our previous report (Kobayashi and Odake 2017), the *S. pastorianus* suspension was prepared by addition of

S. pastorianus cells (NBRC11027) in 10 L of physiological saline (PS) containing 5% ethanol concentration (approximately 1.0×10^6 CFU/mL).

The MBCO₂ equipment and the treatment procedure

The MBCO₂ treatment was performed using the same equipment as in a previous report (Kobayashi and Odake 2017) and at the following conditions: the temperature, pressure and exposure time in the mixing vessel were set to 10 °C, 1 or 2 MPa, and 5 min, respectively, and the temperature, pressure and exposure time in the heating coil were set to 29–43 °C, 4 MPa, and 1 min, respectively. The temperature in the heating coil increased until a 6-log reduction was achieved.

Thermal treatment

The thermal treatment was performed at 29–51 °C for 1 min using only the heating coil of MBCO₂ equipment. The temperature during the thermal treatment increased until a 6-log reduction was achieved.

Measurement of the number of the surviving *S. pastorianus* cells

The number of surviving *S. pastorianus* cells was measured as follows: 1 mL of a sample or diluted sample was incubated with yeast and mould agar (YMA, Difco, Becton-Dickinson, Franklin Lakes, NJ, an optimum agar), YMA adding 2.5 g/L sodium chloride (YMAS) and yeast nitrogen base agar (YNBA, Difco, a minimum agar) plates, respectively. The sodium chloride concentration of YMAS was decided to 2.5 g/L which was the maximum concentration that injured *S. pastorianus* cells can grow (data not shown). After incubating the plates at 25 °C for 48 h, the plates with 30–300 CFU were chosen, and the colonies were counted. If there were low numbers of viable cells, the colonies on the plates from the undiluted sample were counted (Kobayashi and Odake 2018). The detection limit was 1 CFU/mL.

Measurement of fluorescence polarization (FP) of *S. pastorianus*

The FP of *S. pastorianus* cells was measured by reference to the method of Liao et al. (2010). Forty microliters of 2.0×10^{-3} mol/L 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma-Aldrich Co., St. Louis, USA) solution (in tetrahydrofuran) was added to 10 mL of *S. pastorianus* suspension (1.0×10^9 CFU/mL). The mixture was incubated in a water bath at 30 °C for 1 h, washed twice by centrifugation

(5 °C, 10,000×g, 30 min) with PS and re-suspended in 10 L of PS containing 5% ethanol concentration. The suspension was subjected to MBCO₂ treatment and an aliquot of the treated suspension was measured with a fluorescence spectrophotometer (RF-5300PC, Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 358 nm and an emission wavelength of 410 nm. The results were expressed as a relative ratio of FP calculated by the following equation:

$$\begin{aligned} &\text{Relative ratio of FP} \\ &= (\text{fluorescence intensity after treatment} \\ &\quad - \text{fluorescence intensity before treatment}) \\ &\quad / \text{fluorescence intensity before treatment} \end{aligned} \quad (1)$$

Measurement of alkaline phosphatase (AP) activity of *S. pastorianus*

Briefly, 750 µL of the *S. pastorianus* suspension, concentrated tenfold by centrifugation (5 °C, 10,000×g, 30 min), was mixed with 750 µL of 7.0×10^{-3} mol/L *p*-nitrophenolphosphate solution (in 1.0×10^{-3} mol/L glycine buffer, pH 10.5) and incubated at 37 °C for 4 h. Then, 1.5 mL of 1 mol/L sodium hydroxide solution was added to the solution to stop the reaction and the absorbance at 405 nm was measured with a spectrophotometer (U-5100, Hitachi High-Tech Science Co., Tokyo, Japan). The results were expressed as a relative ratio of AP activity calculated by the following equation:

$$\begin{aligned} &\text{Relative ratio of AP activity} \\ &= (\text{absorbance after treatment} \\ &\quad - \text{absorbance before treatment}) \\ &\quad / \text{absorbance before treatment} \end{aligned} \quad (2)$$

Measurement of intracellular pH of *S. pastorianus*

The intracellular pH (pH_{in}) of *S. pastorianus* was measured with 5(6)-carboxyfluorescein diacetate succinimidyl ester (Dojindo Laboratories, Kumamoto, Japan) and a fluorescence spectrophotometer (RF-5300PC, Shimadzu Co.) according to the report of Kobayashi and Odake (2017).

Statistical analysis

All experiments were performed in triplicate. The data represent the means with standard errors of the results of the triplicate experiments. The significant differences were evaluated by the Tukey–Kramer test ($p < 0.05$).

Results and discussion

Inactivation efficiency

The inactivation of *S. pastorianus* by MBCO₂ and thermal treatments is shown in Fig. 1. The effect of MBCO₂ on the inactivation of *S. pastorianus* cells was significantly higher than that of thermal treatment and the number of surviving *S. pastorianus* cells decreased with increasing pressure in the mixing vessel or temperature in the heating coil of MBCO₂, because a 6-log reduction was achieved at temperatures in the heating coil of 51, 43 and 37 °C at thermal treatment and MBCO₂ at 1 and 2 MPa of pressure in the mixing vessel, respectively. The results showed the same tendency as in the previous reports (Garcia-Gonzalez et al. 2007; Kobayashi and Odake 2017, 2018). The number of surviving *S. pastorianus* cells treated by MBCO₂ with the mixing vessel at 2 MPa decreased from the temperature in the heating coil of 33 and 35 °C by measurement with both YMAS and YNBA, and with YMA, respectively. In addition, inactivation of *S. pastorianus* by MBCO₂ with the mixing vessel at 1 MPa occurred from the temperature in the heating coil of 39 and 41 °C by measurement with both YMAS and YNBA, and with YMA, respectively. Conversely, the number of surviving *S. pastorianus* cells after thermal treatment decreased from the temperature in the heating coil of 41 and 43 °C by measurement with YNBA, and with both YMAS and YMA, respectively. Previously, it was documented that the membrane damage of *S. pastorianus* cells was induced from the temperature more than 40 °C (Kobayashi and Odake 2017, 2018). Hong and Pyun (2001) noted that the number of surviving *Lactobacillus plantarum* cells treated with pressurized CO₂ at 30 °C and 5 MPa measured with de Man Rogosa and Sharpe (MRS) agar was higher than that with MRS agar adding 3 g/L sodium chloride and more than 90% survivors has been injuring. In addition, Wu et al. (2007) measured the number of surviving *E. coli* cells treated by pressurized CO₂ at 35 °C and 7.8 MPa by agar adding sodium chloride and suggested that more than 90% survivors on agar without sodium chloride were subjected to injury on the cell membrane. Therefore, it was confirmed that most of *S. pastorianus* cells was injured by MBCO₂ at a temperature less than 40 °C and by thermal treatment at a temperature more than 40 °C.

Fluorescence polarization (FP)

The FP of *S. pastorianus* cells after MBCO₂ and thermal treatments is shown in Fig. 2. The FP of *S. pastorianus* cells treated by MBCO₂ increased with increasing the

Fig. 1 Inactivation of *S. pastorianus* cells by MBCO₂ and thermal treatments. **A** Measurement by YMA, **B** measurement by YMAS, **C** measurement by YNBA. Triangle: thermal treatment, square: MBCO₂ treatment with the mixing vessel pressure at 1 MPa, circle: MBCO₂ treatment with the mixing vessel pressure at 2 MPa. Different letters indicate significant differences in each treatment by the Tukey–Kramer method ($p < 0.05$)

temperature in the heating coil, equally at pressure in the mixing vessel of 1 and 2 MPa. On the other hand, the FP of *S. pastorianus* cells increased significantly from 41 °C by

Fig. 2 Fluorescence polarization (FP) of *S. pastorianus* cells treated by MBCO₂ and thermal treatments. **A** Thermal treatment, **B** MBCO₂ treatment with the mixing vessel pressure at 1 MPa, **C** MBCO₂ treatment with the mixing vessel pressure at 2 MPa. Different letters indicate significant differences in each treatment by the Tukey–Kramer method ($p < 0.05$)

thermal treatment. The gel-fluid phase transition of aqueous dipalmitoylphosphatidylcholine liposomes occurred at 42 °C (Bothun et al. 2005). Temperature increase will decrease the physical stability of the membrane, adopting a crystalline liquid appearance (Barba et al. 2017). Therefore, MBCO₂ caused the phase transition of the cell membrane of *S. pastorianus*. Liao et al. (2010) and Li et al. (2013) recognized that pressurized CO₂ increased FP of *E. coli* and *S. cerevisiae* cells measured with DPH, indicating the rigidity increase and the fluidity decrease of the cell membrane. The change in FP induced by MBCO₂ with the mixing vessel at 2 MPa agreed with the number of surviving *S. pastorianus* cells measured with YMAS and YNBA but not at 1 MPa. Therefore, it was revealed that membrane fluidity of *S. pastorianus* cells was influenced by thermal treatment at a temperature higher than 40 °C but became sensitive to less than 40 °C by using the MBCO₂.

Alkaline phosphatase (AP) activity

The AP activity of *S. pastorianus* cells after MBCO₂ and thermal treatments is shown in Fig. 3. The AP activity of *S. pastorianus* cells increased gradually from 39 °C by thermal treatment, reached a maximum at 47 °C and then decreased drastically with further increasing temperature. On the other hand, the AP activity of *S. pastorianus* cells treated by MBCO₂ with the mixing vessel at 1 MPa increased with increasing temperature in the heating coil, peaked at 39 °C and then decreased with further increasing temperature. Conversely, AP activity of *S. pastorianus* cells treated by MBCO₂ with the mixing vessel at 2 MPa showed a slight increase at the temperature in the heating coil of 31 °C, and significantly decreased by further increasing temperature. Therefore, it was recognized that the AP activity of *S. pastorianus* cells increased by MBCO₂ and thermal treatments correlated highly with the FP but decreased by further increasing temperature. The AP activity of *S. pastorianus* cells after MBCO₂ and thermal treatments might not be associated with the inactivation efficiency, because the AP activity reached a maximum when the survivors decreased. The correlativity between inactivation of AP and bacteria by pressurized CO₂ has been previously reported (Li et al. 2012; Bertoloni et al. 2006). Bertoloni et al. (2006) also demonstrated that the release of AP from *E. coli* caused by pressurized CO₂ led to the reduction in the enzyme activity. In contrast, Kim et al. (2007) reported that the AP of *E. coli* was activated by pressurized CO₂. The AP of *S. pastorianus* may be activated due to the disengagement from the periplasm (Nikerson et al. 1948), associated with the change in the cell membrane fluidity by pasteurization (Tsuchido et al. 1985). It was also considered that inactivation of AP by

Fig. 3 Relative ratio of alkaline phosphatase (AP) activity of *S. pastorianus* cells treated by MBCO₂ and thermal treatments. **A** Thermal treatment, **B** MBCO₂ treatment with the mixing vessel pressure at 1 MPa, **C** MBCO₂ treatment with the mixing vessel pressure at 2 MPa. Different letters indicate significant differences in each treatment by the Tukey–Kramer method ($p < 0.05$)

MBCO₂ with the mixing vessel at 2 MPa could be achieved at a lower temperature than that at 1 MPa because of the greater effect on enzyme inactivation with higher pressure.

Intracellular pH (pH_{in})

The pH_{in} of *S. pastorianus* treated with MBCO₂ is shown in Fig. 4. The pH_{in} of *S. pastorianus* treated by MBCO₂ with the mixing vessel at 1 and 2 MPa decreased significantly from the temperature in the heating coil of 43 and 33 °C, respectively. In the MBCO₂ with the mixing vessel at 2 MPa, the pH_{in}-lowering was consistent with the decrease in the survivors of *S. pastorianus* measured with YMA. Therefore, this result sustained the previous statement that inactivation of *S. pastorianus* by MBCO₂ at 40 °C might be due to intracellular acidification (Kobayashi and Otake 2017), or it was considered that the pH_{in}-lowering resulted from the penetration of dissolved CO₂ or extracellular H⁺ due to the change in cell membrane fluidity associated with the death of *S. pastorianus* by MBCO₂. Conversely, the pH_{in}-lowering was a main factor of the inactivation of *S. pastorianus* by MBCO₂ with the mixing vessel at 1 MPa and was caused due to only the temperature increase to more than 40 °C according to a previous report (Kobayashi and Otake 2018), because the temperature in the heating coil for inactivating *S.*

Fig. 4 Intracellular pH (pH_{in}) of *S. pastorianus* treated by MBCO₂. Square: MBCO₂ treatment with the mixing vessel pressure at 1 MPa, circle: MBCO₂ treatment with the mixing vessel pressure at 2 MPa. Different letters indicate significant differences in each treatment by the Tukey–Kramer method ($p < 0.05$)

pastorianus was lower than that for lowering the pH_{in}. The relationship between pH_{in} and temperature increase on the microbial inactivation by pressurized CO₂ have been never reported besides our studies. Watanabe et al. (2005) also indicated the penetration of extracellular H⁺ into *S. cerevisiae* cells by pressurized CO₂. Therefore, the marked injury of the cell membrane of *S. pastorianus* induced by MBCO₂ with the mixing vessel at 2 MPa allowed extracellular H⁺ to penetrate inside the cells but at 1 MPa was not as severe as to cause the penetration of extracellular H⁺.

Conclusion

In this study, the change of the cell membrane fluidity of *S. pastorianus* by MBCO₂ was revealed to be caused at lower temperature than thermal treatment and considered to be due to the dissolved CO₂. It suggested that the extent differed between the pressures in the mixing vessel at 1 and 2 MPa, because the penetration of extracellular H⁺ inside the cells was induced by MBCO₂ with the mixing vessel at 2 MPa but not 1 MPa.

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