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Effect of pressure on the inactivation of enzymes and *hiochi* bacteria in unpasteurized sake by low-pressure carbon dioxide microbubbles

Fumiyuki Kobayashi [a, *], Sachiko Odake [a], Ken Kobayashi [b], Hiroshi Sakurai [b]

[a] Faculty of Applied Life Science, Nippon Veterinary and Life Science University, 1-7-1 Kyonantyo, Musashino, Tokyo, 180-8602, Japan
[b] Asahishuzo Co. Ltd., 2167-4 Osogoe, Shutomachi, Iwakuni, Yamaguchi, 742-0422, Japan

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ABSTRACT

The effect of pressure in the mixing vessel on the inactivation of enzymes and *hiochi* bacteria and on the change in free amino acid content in unpasteurized sake (UPS) using a two-stage system of low-pressure carbon dioxide microbubbles ($MBCO_2$) was investigated. At a pressure of 0 MPa in the mixing vessel, *hiochi* bacteria in UPS were completely inactivated by heating at 65 °C for 1 s with two-stage $MBCO_2$; α-amylase, glucoamylase and acid carboxypeptidase were inactivated after heating for 5 s, although α-glucosidase activity remained at approximately 13% even after 20 s. However, the inactivation efficiency increased with increasing pressure in the mixing vessel. In addition, the contents of aspartic acid, glutamic acid, alanine and arginine, key free amino acids involved in producing the taste of sake, in UPS tended to decrease by two-stage $MBCO_2$ at all tested conditions, although these were minimally affected by pressure in the mixing vessel and exposure time in the heating coil.

1. Introduction

Sake is a traditional alcoholic beverage in Japan. Certain enzymes and alcohol-philic and alcohol-resistant lactic acid bacteria known as *hiochi* bacteria remain in unpasteurized sake (UPS) after the brewing stage; these bacteria cause degradation of the quality and spoilage of the sake by causing white turbidity, increasing acid content, and generating off-flavors during storage and distribution (Noshiro and Momose, 1970). Accordingly, UPS is typically subjected to heat treatment at approximately 65 °C for 3 min to prevent enzymatic activity and the growth of *hiochi* bacteria. However, heat treatment often causes the loss of fresh flavors in UPS (Yamamoto and Saito, 1989; Tanimoto et al., 2004). Therefore, new techniques for inactivating *hiochi* bacteria and enzymes without affecting the quality of the sake are desired by the sake brewing industry.

To avoid undesirable changes in the taste and flavor of food due to heating, pressurized carbon dioxide (CO_2) has been investigated as a non-thermal pasteurization technique (Damar and Balaban, 2006; Garcia-Gonzalez et al., 2007; Spilimbergo and Bertucco, 2003). In addition, a review on enzyme inactivation by pressurized CO_2 has been published (Hu et al., 2013). However, the loss in flavor and ascorbic acid and changes in color, pH, and turbidity of food have been observed to be induced by pressurized CO_2 (Cappelletti et al., 2015; Chen et al., 2009; Dagan and Balaban, 2006; Da Porto et al., 2010). Recently, we developed a process for heating and pressurizing after feeding CO_2 microbubbles (MB) into a liquid sample in a mixing vessel at a temperature <10 °C and pressure lower than critical pressure (two-stage $MBCO_2$); we reported the inactivation of polyphenol oxidase (PPO), *Fusarium oxysporum* f.sp. *melonis* spore, *Pectobacterium carotovorum* subsp. *carotovorum*, *Lactobacillus fructivorans*, *Saccharomyces cerevisiae*, and *Saccharomyces pastorianus* using this two-stage $MBCO_2$ process (Kobayashi et al., 2013a, b, c; 2014a, b). Furthermore, the quality of the sake treated with two-stage $MBCO_2$ at various temperatures was evaluated. The sake treated with the two-stage $MBCO_2$ at 65 °C received the highest rating on sensory evaluation, and free amino acid content in the sake was affected by the treatment temperature of two-stage $MBCO_2$ (Kobayashi et al., 2014c). However, the effect of pressure on the inactivation of enzymes and *hiochi* bacteria and on the change in free amino acid content in the sake by two-stage $MBCO_2$ has not yet been investigated. It is of practical importance to evaluate the effect of pressure because working pressure is related to an equipment's cost.

Therefore, the aim of the present study was to investigate the

* Corresponding author.
E-mail addresses: fkoba@nvlu.ac.jp (F. Kobayashi), odake@nvlu.ac.jp (S. Odake), k.kobayashi@asahishuzo.ne.jp (K. Kobayashi), webmaster@asahishuzo.ne.jp (H. Sakurai).

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effect of the pressure in the mixing vessel on the inactivation of enzymes and *hiochi* bacteria and on the change in free amino acid content in the sake by two-stage $MBCO_2$.

2. Materials and methods

2.1. UPS

UPS (ethanol concentration 15%) brewed at Asahishuzo Co. Ltd. (Iwakuni, Japan) was used for the experiment. The number of surviving *hiochi* bacteria (*L. heterohiochii* and/or *L. homohiochii*), and α-amylase (AA), glucoamylase (GA), acid carboxypeptidase (AC) and α-glucosidase (AG) activities in the UPS were 1.0×10^5 CFU/mL, and 1.1, 9.9, 9.6×10^2 and 1.2 U/mL, respectively.

2.2. Equipment and procedure for two-stage $MBCO_2$

The experimental equipment used for two-stage $MBCO_2$ in the present study was a modification of the equipment described in a previous report (Kobayashi et al., 2014c). This equipment was constructed by Izumi Kosyo Co. Ltd. (Chigasaki, Japan) based on our design, and was used as follows: 10 L UPS was injected into the mixing vessel (300 mm diameter × 220 mm height; volume 15 L) and was cooled to 5 °C. The top of the vessel was then tightly closed and gaseous CO_2 was fed into the headspace of the mixing vessel to reach the treatment pressure. $MBCO_2$ was generated by sending the mixture of UPS and gaseous CO_2 introduced at 2 L/min to an ejector-type MB generator (Aura-tec Co. Ltd., Kurume, Japan) using a circulating pump (0.75 kW motor pump, Teikoku Electric Mfg. Co. Ltd., Tatsuno, Japan) at 15 L/min until the dissolved CO_2 concentration in the UPS reached saturation at 5 °C and the treatment pressure (5 min). The bubble size of $MBCO_2$ measured in our previous study (Kobayashi et al., 2013a) was 37 μm. However, the relationship between the bubble size of $MBCO_2$ and the pressure in the mixing vessel is unclear. The CO_2-saturated UPS was continuously pumped using a metering pump (Nihon Seimitsu Kagaku Co., Ltd., Tokyo, Japan) into a heating coil (4 mm diameter × 2000 mm length; volume 25 mL). The exposure time in the heating coil was set by adjusting the flow rate of the metering pump. The treated sample was collected from the back pressure valve mounted at the exit of the heating coil. The two-stage $MBCO_2$ treatment was performed under the following conditions: temperature, pressure, and exposure time in the mixing vessel were set at 5 °C, 0, 1, or 2 MPa and 5 min, respectively; temperature, pressure, and exposure time in the heating coil were set at 65 °C, 6 MPa and 1, 5, 10, 15 or 20 s, respectively.

2.3. Measurement of the number of surviving hiochi bacteria

The number of surviving *hiochi* bacteria cells was measured by plating 1 mL of the sample or diluted sample on duplicate plates of S.I. [10 g yeast extract, 5 g polypeptone, 25 g glucose, 0.1 g magnesium sulfate heptahydrate, 0.0025 g manganese (II) sulfate tetrahydrate, 0.0025 g iron (II) sulfate heptahydrate, 0.05 g sodium azide, 10 g sodium acetate, 0.005 g mevalonic acid, and 0.6 g agar; Brewing Society of Japan, Tokyo, Japan], 100 mL ethanol, and 900 mL deionized water (Sugama and Iguchi, 1970), and 15 g agar. The plates were anaerobically incubated at 30 °C for 7 days. After incubation, the number of colonies that had formed on the plates was counted. The detection limit was 1 CFU/mL. All experiments were performed in triplicates. Data are presented as means with standard errors of the results from triplicate experiments.

2.4. Measurement of enzyme activities

The residual activities (RA) of GA and AG in sake were measured using a diastatic power assay kit. The RA of AC and AA in sake were measured using AC and AA assay kits, respectively. All kits were purchased from Kikkoman Co. (Noda, Japan). One unit of AA, GA and AC activities were defined as the amount required to produce 1 μmol of 2-chrolo-4-nitro phenol, 4-nitro phenol and tyrosine in 1 min, respectively (Gomi, 1993). One unit of AG activity was defined as the amount required to produce 1 μmol of 4-nitro phenol in 1 min. The relative RA (RRA) of AG was calculated using the following equation:

$$\text{RRA}\ (\%) = (\text{RA of AG after treatment} / \text{RA of AG before treatment}) \times 100 \quad (1)$$

All experiments were performed in triplicates. Data are presented as means with standard errors of the results from triplicate experiments.

2.5. Inactivation kinetics analysis of AG

Inactivation kinetics of AG were analyzed using a conventional first-order model:

$$\log (A_t / A_0) = (k / 2.303)\, t \quad (2)$$

$$D = 2.303 / k \quad (3)$$

where A_t is the RRA of AG at any time t, A_0 is the initial RRA of AG, k is the reaction rate constant (min^{-1}) in a given condition, and the decimal reduction time (D value) is the exposure time required for 90% inactivation of the initial activity at fixed temperature and pressure conditions.

Z_P represents the pressure increase required for a 90% reduction of D value, which is calculated using the following equation (Zhang et al., 2010):

$$\log (D_1 / D_2) = (P2 - P_1) / Z_P \quad (4)$$

The pressure dependence of k can be expressed using the activation volume (V_a, cm^3/mol), respectively, which are obtained using the Arrhenius equation (Balaban et al., 1991; Gui et al., 2007; Liu et al., 2008, 2010):

$$\ln (k_1/k_2) = V_a/RT\,(P_2 - P_1) \quad (5)$$

where P_2 and P_1 are the pressures that correspond to k_1 and k_2, R is the gas content (8.314 J/mol/K), and T is the absolute temperature (K). The values of Z_P were obtained from the negative slope of the regression line of log D versus P, respectively. V_a value were estimated from the regression line of ln k versus $(1/T)$ and P, respectively.

2.6. Measurement of free amino acid content

Sake and 3% sulphosalicylic acid solution were mixed at a ratio of 1:1 by volume and refrigerated overnight. The mixture was filtered through a 0.22 μm filter. Free amino acids in the filtrate were measured using an automated amino-acid analyzer (JLC500-500/V2, JEOL Ltd., Akishima, Japan). The result was expressed as relative content, calculated using the following equation:

$$\text{Relative content}\ (\%) = (\text{content after treatment} / \text{content before treatment}) \times 100 \quad (6)$$