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Short communication

A combined intervention using fermented ethanol and supercritical carbon dioxide to control *Bacillus cereus* and *Bacillus subtilis* in rice

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ABSTRACT

Although the contamination of rice by *Bacillus* spp. is a great concern to the rice industry, very few studies to date have been performed on the control of *Bacillus* spp. in rice. The elimination of microorganisms in rice is quite difficult due to its solid phase. In this study, a new method of intervention for the inactivation of *Bacillus cereus* and *Bacillus subtilis* in rice was developed using fermented ethanol (FE) and supercritical carbon dioxide (SC–CO_2). Raw rice samples inoculated with *Bacillus* spp. were treated with 1. FE only (concentrations: 0, 10, 30, 50, and 70%; treatment duration: 5, 10, 20, 30, and 60 min), 2. SC–CO_2 only (temperatures: 36, 40, and 44 °C; pressure: 100, 150, and 200 bar; treatment duration: 10, 20, and 30 min) or 3. with both FE (30% for 30 and 60 min) and SC–CO_2 (36 °C, 100 bar, 10 min and 44 °C, 200 bar, 30 min). The survival of *Bacillus* spp. populations was assayed, as were any changes in the hardness and color of the treated rice. When rice samples were treated with the combined treatment of FE (30%, 60 min) and SC–CO_2 (200 bar, 44 °C, 30 min), *Bacillus* spp. were maximally reduced by approximately 5 log CFU/g. Sufficient log reduction of *B. cereus* (4.76 log CFU/g) and *B. subtilis* (4.66 log CFU/g) were also obtained with mild treatment (FE: 30%, 30 min and SC–CO_2: 100 bar, 36 °C, 10 min). The combined treatment led to beneficial changes including decrease in the hardness of cooked rice and whitening of rice (increase of lightness and decrease of yellowness) compared to that of untreated rice, resulting in an increase in perceived quality. The changes of hardness and lightness were mainly affected by FE and SC–CO_2, respectively. Our results indicate that a combined treatment with FE and SC–CO_2 can be successfully used for the inactivation of *Bacillus* spp. in rice, with simultaneous improvements in rice.

1. Introduction

Rice is a staple food in the Asian diet. The production and consumption of rice-based products including noodles, cakes, snacks, gruels, beverages, and alcoholic fermented beverages, have not only grown but become more diverse in recent decades. However, among the greatest concerns of the rice industry is the fact that rice can be frequently contaminated with microorganisms during the cultivation, harvesting, handling and processing stages (Haque & Russell, 2005). Previous research has shown that raw rice, cooked rice and rice-based products, such as noodles and pasta, are frequently contaminated with *Bacillus cereus* (Fangio, Roura, & Fritz, 2010; Nichols, Little, Mithani, & De Louvois, 1999; Sarrias, Valero, & Salmeron, 2002; Yang, Tao, Liu, & Zhu, 2008), an opportunistic human pathogen that causes two types of food-borne diseases: a diarrheal syndrome caused by complex enterotoxins (diarrheal toxin) and emetic syndrome caused by emetic toxins. Recently, contamination of food products (including rice) with *Bacillus subtilis* has also been shown to underlie food-borne diseases in humans (Fangio et al., 2010; From, Pukall, Schumann, Hormazabal, & Granum, 2005).

Although extremely important, very few studies to date have focused on the control and suppression of contamination by *Bacillus* spp. in rice (Sarrias, Valero, & Salmeron, 2003) and those included only the use of washing methods in food industry. Because the quality of rice can be easily changed under high temperatures or pressure conditions, thermal treatments cannot be efficiently employed to rice. In addition, the elimination of microorganisms from rice is further complicated by its solid phase.

Given the increasing demand for safe and fresh food, the development of new intervention methods, such as non-thermal treatments, is necessary both to ensure microbial safety and maintain the natural appearance and quality of food products. Fermented ethanol (FE) is an edible and natural form of ethanol, an

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effective antimicrobial agent used for the control of microorganisms such as *Staphylococcus aureus* (Shapero, Nelson, & Labuza, 1978), *Listeria monocytogenes* (Oh & Marshall, 1993) and *Salmonella typhimurium* (Mies et al., 2004). Ethanol has also been widely used as a bactericidal agent in various industries. The supercritical carbon dioxide (SC–CO_2), a supercritical fluid with several attractive properties such as low cost, non-toxicity, and chemical stability (Sauceau, Nikitine, Rodier, & Fages, 2007), has also been used as an antibacterial agent in various foods, including meat (Choi, Bae, Kim, Kim, & Rhee, 2009), apple juice (Bae, Lee, Kim, & Rhee, 2009), sprout (Jung, Choi, & Rhee, 2009), and whole milk (Erkmen, 1997), and has been considered an attractive non-thermal treatment (Kim, Kim, & Rhee, 2010).

Although FE and SC–CO_2 have been applied to various foods to eliminate bacterial contamination, their effect on rice contaminated by *Bacillus* spp. has not yet been studied. In this study, the isolated and combined effect of FE and SC–CO_2 in the control of *Bacillus* spp. populations in rice was investigated. Additionally, potential changes on the innate properties of rice (hardness and color) caused by the combined treatment were also examined.

2. Materials and methods

2.1. Test organisms

The three strains of *B. cereus* (ATCC 10876, 11778, 14579) and *B. subtilis* (ATCC 9372, 15245, 21336) used in this study were obtained from the Food Microbiology Culture Collection at Korea University (Seoul, Korea). They were maintained on tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD) slants at 4 °C and subcultured at monthly intervals. Each strain of *B. cereus* and *B. subtilis* was incubated in tryptic soy broth (TSB; Difco) at 30 and 37 °C, respectively, for 24 h. Enriched cell suspensions were centrifuged at 2600*g* for 15 min (Centra–CL2, IEC, Needham Heights, MA) and were washed twice with 0.2% sterile peptone water. The final pellets were resuspended in sterile 0.2% peptone water.

2.2. Sample preparation and inoculation

Raw rice (*Oryzae sativa* L.) was purchased at a local retail store. The rice sample was spread on aluminum foil and exposed to UV overnight in a laminar flow biological safety cabinet. To ensure the absence of *Bacillus* spp., 25 g of rice sample were placed into stomacher bags containing 225 ml of 0.2% sterile peptone water and homogenized at 260 rpm for 2 min with a stomacher (400 Circulator, Seward, London, UK). Two hundred microliters of tenfold diluents (10^{-1}) were spread-plated on five plates of TSA (detection limit: 10 CFU/g). The plates were incubated for 24 h at 30 and 37 °C for *B. cereus* and *B. subtilis*, respectively. Following incubation, there was no *Bacillus* spp. detected. The inoculation of *B. cereus* and *B. subtilis* in rice was conducted as described by Sarrias et al. (2003). The raw rice (100 g) was placed into a sterile stomacher bag and 1 ml of cocktailed cultures (the concentration of inoculums was 8–9 log CFU/ml) of *B. cereus* or *B. subtilis* prepared with the above mentioned method was sprayed uniformly onto rice sample with a sterile spray gun, yielding a concentration of approximately 10^6–10^7 CFU/g. The inoculated sample was dried in a laminar flow biological safety cabinet at room temperature for 4 h to allow sufficiently attachment of bacteria onto raw rice sample.

2.3. Fermented ethanol treatment

FE (Daehan Ethanol Life Ltd., Seoul, Korea) was diluted with sterile distilled water to concentrations of 10, 30, 50, and 70% (v/v). Ten grams of the inoculated rice samples were placed into sterile bags containing 100 ml of FE at each of the FE concentrations. Each sample was treated at room temperature for 0, 5, 10, 30, and 60 min. Samples treated with sterile distilled water (0% FE) served as controls. After the FE treatment, samples were washed with 100 ml of sterile distilled water in a shaking water bath (Vision Sci. Co. Ltd., Incheon, Korea) at 100 rpm for 2 min. All treatments were replicated three times.

2.4. Supercritical carbon dioxide treatment

The SC–CO_2 treatment was conducted as described by Kim et al. (2010). A schematic diagram of the SC–CO_2 system (Ilshin Co., Daejeon, Korea) used in the present study is shown in Fig. 1. Rice samples (10 g) inoculated with *B. cereus* or *B. subtilis* were placed into a sterile conical tube and loaded into the vessels of SC–CO_2 operator. When the temperature of the SC–CO_2 operator reached the set temperature, the CO_2 pump switched on and the inlet valve was opened to allow CO_2 to inject into the vessel (the decompression valve was locked during injection). When the pressure reached the designated pressure, the CO_2 pump was switched off and the inlet valve was closed for the duration of the treatment time. After the sample was treated, the decompression valve was slowly opened to decompress the pressure in the vessel below atmospheric pressure. The SC–CO_2 treatments were performed in triplicate under various conditions of air pressure (100, 150, and 200 bar), temperature (36, 40, and 44 °C), and time (10, 20, and 30 min).

2.5. Combination treatment with fermented ethanol and supercritical carbon dioxide

FE treatments (concentration of 30% treated for 30 min and 60 min) were applied prior to the supercritical carbon dioxide treatments (100 bar for 10 min at 36 °C and 200 bar for 30 min at 44 °C). The specific conditions used in each treatment were determined based on the results of the FE and SC–CO_2 experiments previously described. In the case of the FE treatment, a concentration of 30% showed a relatively high bacterial reduction, at the same time being attractive in terms of its associated cost and applicability in the industry. For the SC–CO_2 treatment, the conditions chosen were those corresponding to the lowest and highest possible CO_2 critical points that caused changes in the rice properties (similar to those observed during cooking) when the temperature was increased.

2.6. Bacterial enumeration

After treatment, samples (10 g) were placed into a stomacher bag containing 90 ml of sterile 0.2% peptone water and

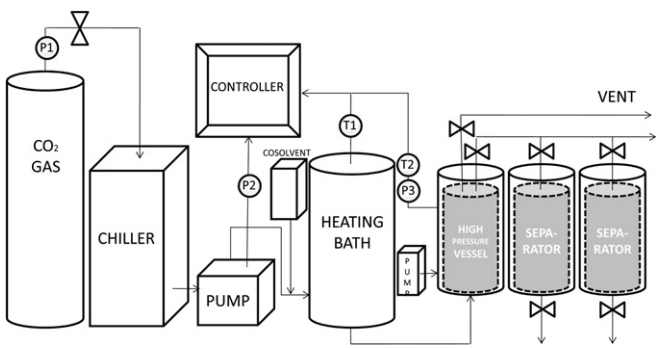

Fig. 1. A schematic diagram of the supercritical carbon dioxide system. T1 and T2 indicate inlet and vessel temperature, respectively. P1, P2, and P3 indicate the outlet of the CO_2 cylinder, the inlet, and the vessel pressure, respectively.