



Disinfection of wheat grains contaminated with *Penicillium oxalicum* spores by a supercritical carbon dioxide-water cosolvent system

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ABSTRACT

Spores of the plant pathogenic fungus *Penicillium oxalicum* inoculated onto wheat grains were inactivated using supercritical carbon dioxide (SC-CO₂). After the SC-CO₂ treatment at various conditions of temperature, time and amount of water cosolvent, the colony forming units (CFU) of fungal spores on wheat grains and the germination yields of wheat grains were determined. Among these SC-CO₂ treatment parameters, the inactivation of *P. oxalicum* spores was found to be significantly increased by adding water cosolvent. The optimal conditions determined by ridge analysis of response surface methodology were 233 μ L water, 44 °C, and 11 min, which resulted in a 6.41 log₁₀ CFU reduction of *P. oxalicum*. However, the germination yields of wheat grains significantly decreased when water cosolvent of 150 or 300 μ L was added to the grains contained in the 100 mL SC-CO₂ treatment vessel.

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1. Introduction

Wheat is cultivated worldwide as a food resource, and most wheat grains are stored for certain periods prior to human and animal consumption. The stored wheat grains are susceptible to contamination by various fungi even under general storage conditions (Tabuc et al., 2009), which can cause serious deterioration in quality and quantity of the grains, ultimately resulting in significant economic loss. For example, more than 400 million tons of grains are stored every year, and grains damaged by insects and fungi during storage amount to over 500 million dollars in the US alone (Scott, 1991). In addition, some mycotoxins produced by various fungi as secondary metabolites are known to be lethal to both humans and animals (Dagenais and Keller, 2009; Jestoi, 2008). *Penicillium* species are among the most common fungi found in daily life, and they are known to cause the blue mold disease in crop seeds, fruits, and vegetables during transport and storage after harvest (Palou et al., 2001; Ritieni, 2003). Mycotoxins such as ochratoxin and patulin produced by *Penicillium* spp. have been found to be carcinogenic to humans (Moake et al., 2005; Schwartz, 2002). Therefore, a disinfection or sterilization process for grains before storage would be advantageous.

Various techniques have been used to decontaminate or sterilize seed, including physical or chemical treatments using heat (Rustom, 1997), chemical agents (Fett, 2002), high hydrostatic pressure

(Kadlec et al., 2006), pulsed UV-light (Jun et al., 2003), gamma-irradiation (Braghini et al., 2009), and ozone (Wu et al., 2006). However, these methods may cause problems such as destruction of nutrients, environmental pollution, high operating cost, and low germination ratio.

Supercritical carbon dioxide (SC-CO₂) has been evaluated as an alternative milder thermal sterilization method. SC-CO₂ has been shown to eliminate bacterial cells (Kamihira et al., 1987; Kim et al., 2009b, 2008, 2007), microbial biofilms (Mitchell et al., 2008; Mun et al., 2009), and bacterial spores (Watanabe et al., 2003; Zhang et al., 2007). In SC-CO₂, not only pressure and temperature but also a cosolvent are the major factors that determine the physico-chemical properties of the supercritical fluid. Upon adding a polar cosolvent to CO₂, the polarity of SC-CO₂ can be significantly increased (Dobbs et al., 1987). In previous studies, fungal spores were more susceptible to SC-CO₂ modified with a polar cosolvent such as water or ethanol (Kamihira et al., 1987; Park et al., 2012).

To date, no previous study has used a real food system to evaluate the effect of SC-CO₂ on fungal spores, which are generally more resistant to physical or chemical inactivation methods than vegetative bacteria. In particular, fungal spores are more resistant to heat, pressure, and extreme pH than bacterial cells. The objective of this work was to investigate the effects of SC-CO₂ on *Penicillium oxalicum* spores inoculated on wheat and the germination yields of those SC-CO₂-treated grains. The inactivation conditions for fungal spores were optimized using the Box–Behnken design of response surface methodology (RSM), and the germination yields of wheat grains were evaluated after SC-CO₂ treatment. This is the first

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report of disinfection of fungi in a real food system using SC-CO₂ with a cosolvent.

2. Materials and methods

2.1. Grains and fungal strain

Wheat grains were harvested from the Korea University Farm (Deokso, Korea) in 2007 and were stored at 4 °C until use. The moisture content of wheat grains was $3.84 \pm 0.1\%$ (w/w). *P. oxalicum* KACC 41013 was obtained from the Korea Agricultural Culture Collection (KACC; Suwon, Korea) in April, 2007.

2.2. Preparation of spore suspension

P. oxalicum was inoculated on Potato Dextrose Agar (Difco, Detroit, MI, USA), and the agar medium was incubated at 25 °C for 7 days until the colonies were fully sporulated. The spores were transferred to a sterile tube using a cork borer and vortexed with 10 mL sterile water for 5 min. The initial concentration of *P. oxalicum* spores was approximately 1×10^7 CFU/mL.

2.3. Inoculation of grains

Three grams of wheat grains were inoculated with 3 mL of spore suspension and mixed for 2 min. After inoculation of the spores onto the grains, the grains were placed on sterile gauze and air dried in a laminar flow hood (Biofree, Seoul, Korea) overnight. The initial concentration of spores on the inoculated grains was approximately 10^6 CFU/g. The initial spore concentration was confirmed by plating onto PDA in triplicate.

2.4. SC-CO₂ treatment

The SC-CO₂ treatment system (Ilshin Autoclave, Daejeon, Korea) shown in Fig. 1 was used for the SC-CO₂ treatment of spores on the grains. After the SC-CO₂ treatment vessel reached a set temperature, 3 g of wheat (approximately 70 grains) were transferred to a sterile tube, and the predetermined amount of cosolvent water was added to the grains using a pipette, and then the loosely capped sterile tube was loaded into the SC-CO₂ treatment vessel. Liquid CO₂ (purity of 99.5%, Daehan Specialty Gases, Seoul, Korea) was pumped into the SC-CO₂ treatment vessel until the pressure reached the set value. SC-CO₂ treatment was conducted in a static mode generally by keeping the vent values, V-2 and V-3, closed for the treatment period. After the treatment, the SC-CO₂ treatment vessel was slowly depressurized

by opening V-3 to prevent the grain surface from being cracked by abrupt depressurization. All samples were treated in triplicate.

2.5. Enumeration of viable spores after SC-CO₂ treatment

After SC-CO₂ treatment, SC-CO₂ treated grains were transferred to a sterile tube, and 3 mL sterile water was added to the tube. The sample was then vortexed for 2 min. The number of viable spores was quantified by spreading 0.1 mL of serially-diluted samples onto PDA agar in triplicate, and the agar plates were then incubated at 25 °C for 72 h. The mean viable spore number was divided by the mean of the initial spore numbers, and the data was expressed in log₁₀.

2.6. Evaluation of germination yield of SC-CO₂-treated grains

The untreated and grains treated by SC-CO₂ were placed on sterile filter paper (a diameter of 110 mm, No. 2; Whatman, Brentford, UK) in a Petri dish (SPL Lifescience, Pocheon, Korea) containing distilled water. The Petri dish containing the grains were kept in an incubator (MIR-154, SANYO North America, San Diego, CA, USA) at 25 °C, and the filter paper was kept moist to allow for germination. After 3 days, grains that grew longer than 3 mm were counted as germinated grains.

2.7. Experimental design

The Box–Behnken design was used to determine the optimal conditions for inactivating the fungal spores on grains (Myers and Montgomery, 1995). In our preliminary experiments, SC-CO₂ pressure did not significantly affect the inactivation yield of fungal spores in the supercritical region up to 250 bar. Therefore, for the Box–Behnken design in this study, pressure was fixed at 100 bar, and the amount of cosolvent (i.e. water), the treatment temperature, and the treatment time were selected as the independent variables at three levels. The levels were designated low, medium, and high and coded −1, 0, and +1, respectively as shown in Table 1, and the dependent variable was the log₁₀ reduction of CFU of fungal spores. The ranges of these independent variables were determined based on our preliminary experiments. To investigate the effect of cosolvent, 0, 150, and 300 µL of water were added to wheat grains to be treated in the treatment vessel with an internal volume of 100 mL. The SC-CO₂ treatment temperature and treatment time were 40, 45, and 50 °C and 10, 20, and 30 min, respectively.

A statistical program from the SAS software (version 9.0, Raleigh, NC, USA) was used for the response surface regression (RSREG) and

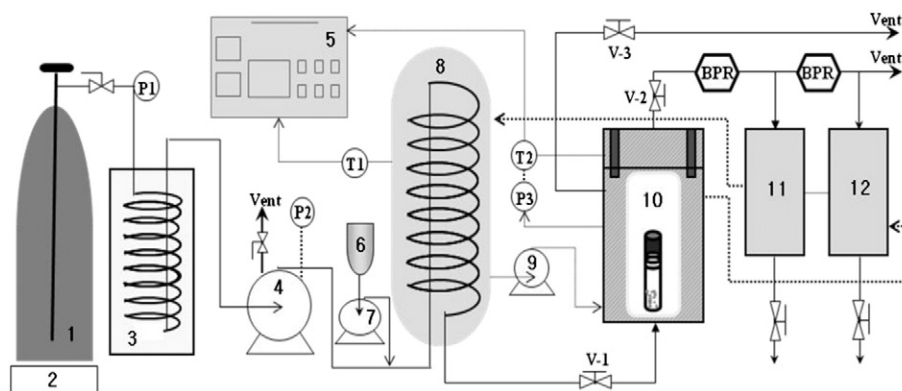


Fig. 1. Schematic diagram of supercritical CO₂ treatment system: 1, CO₂ cylinder; 2, electronic scale; 3, chiller; 4, CO₂ pump; 5, temperature controller; 6, cosolvent reservoir; 7, cosolvent pump; 8, heating bath; 9, circulation pump; 10, treatment vessel; 11, separator 1; 12, separator 2; V1, valve 1; V2, valve 2; BPR, back pressure regulator; dotted lines, water; solid lines, CO₂.

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